NEUCIR SZINWELSKI

DIVERSIDADE DE GRILOS (ORTHOPTERA: GRYLLOIDEA): ASPECTOS ECOLÓGICOS E METODOLÓGICOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 18 de janeiro de 2013.

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Prof. Og Francisco Fonseca de Souza
(Coorientador)

Prof[a]. Karla Suemy Clemente Yotoko

Prof. Marcos Gonçalves Lhano

Prof. José Henrique Schoereder
(Presidente da banca)

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Luis Batarda G.

Sumário

RESUMO

SZINWELSKI, Neucir, D.Sc., Universidade Federal de Viçosa, janeiro de 2013. **Diversidade de grilos (Orthoptera: Grylloidea): Aspectos ecológicos e metodológicos.** Orientador: Carlos Frankl Sperber. Coorientadores: José Henrique Schoereder e Og Francisco Fonseca de Souza.

A teoria ecológica propõe uma série de mecanismos que podem afetar a diversidade. Dentre tantas possibilidades, o objetivo central dessa tese foi investigar a resposta da riqueza de espécies de grilos ao tempo de regeneração florestal e a disponibilidade de recursos. No primeiro capítulo dessa tese, testou-se a resposta da riqueza espécies e composição ao tempo de regeneração florestal, avaliando-se a porcentagem de cobertura de dossel e a profundidade da serrapilheira como variáveis ambientais. A riqueza de espécies aumentou de forma assintótica ao tempo de regeneração florestal e linearmente a porcentagem de cobertura de dossel e a profundidade da serrapilheira. A porcentagem de cobertura de dossel aumentou linearmente ao tempo de regeneração, enquanto que a profundidade da serrapilheira aumentou de forma assintótica. A composição de espécies diferiu completamente entre os fragmentos amostrados. Este trabalho mostrou a importância de se considerar a composição de espécies em estudos que avaliam a resposta da riqueza de espécies a algum distúrbio ambiental. Foi observado que mesmo após a riqueza de espécies atingir a estabilidade, mudanças na composição de espécies continuam ocorrendo. Além disso, o aumento da riqueza de espécies ao tempo de regeneração e às variáveis ambientais, pode, provavelmente, ser reflexo de mudanças e melhorias nas condições e aumento na disponibilidade de recursos necessários, especialmente para espécies mais susceptíveis. Concluiu-se que a recuperação da diversidade de grilos envolve um aumento da complementaridade de nichos em conjunto com alterações na composição de espécies. No segundo capítulo, testou-se a hipótese de que o aumento na quantidade de recursos promoveria aumento na riqueza de espécies, alteraria a composição de espécies e reduziria a equidade da comunidade. A riqueza de espécies foi maior quando o recurso foi adicionado, mas não houve diferença entre os níveis de recursos. Houve mudanças na composição de espécies e interação entre a identidade das espécies e a quantidade de recursos (dois níveis: "sem adição" *vs.* "com adição"). Houve diminuição na equidade da comunidade com a adição de recurso. Embora onívoros, os resultados permitem inferir que a diversidade de grilos é regulada pela quantidade de

recursos. Como descrito na literatura, esses organismos dependem de algumas fontes específicas de recurso, como açucares, para complementar sua dieta. A adição de recursos promove agregação dos indivíduos de espécies raras alterando a estrutura da comunidade e diminuindo a equidade da comunidade. O terceiro e quarto capítulos foram resultados de observações de campo e testes de laboratório. Durante coleta de dados observou-se que algumas espécies de grilos, embora abundantes na serrapilheira, não eram capturadas nas armadilhas que continham a solução matadora padrão, proposta em 2003, para amostragem de grilos. Testes laboratoriais para estudos de biologia molecular também mostraram que a solução padrão degradava rapidamente o DNA. Por isso, foi proposto a substituição da solução padrão por uma nova solução matadora, o álcool combustível. No terceiro capítulo da tese, testou-se a eficiência do álcool combustível como solução matadora e sua capacidade de preservar o DNA. Com a utilização do álcool combustível capturou-se maior número de indivíduos e espécies, e este foi eficiente na preservação do DNA. Além disso, o álcool combustível é mais barato, logisticamente adequado, pois é fácil de ser encontrado, sustentável e não tóxico. Mas porque o álcool combustível coletou maior número de indivíduos e espécies? Essa pergunta foi respondida no quarto capítulo. Testou-se a hipótese de que a maior taxa de captura está relacionada a atração ou devido a redução da fuga. Além de não atrair, no álcool combustível os indivíduos afundam e morrem mais rápido do que na solução padrão, reduzindo a chance de fuga e justificando o aumento na eficiência amostral. A atratividade seria um problema para estudos ecológicos, pois impossibilitaria identificar quais organismos vivem no local e quais organismos se deslocaram de outros locais, atraídos pela substância, ou impossibilitaria comparações com estudos anteriores. Dessa forma, além de trabalhos taxonômicos, anatômicos e moleculares o álcool combustível pode ser utilizado para estudos ecológicos, sem qualquer interferência da solução na amostragem de grilos. A partir da publicação desses trabalhos, passou-se a utilizar o álcool combustível como solução padrão para a amostragem de grilos.

ABSTRACT

SZINWELSKI, Neucir, D.Sc., Universidade Federal de Viçosa, January, 2013. **Cricket diversity (Orthoptera, Grylloidea): Ecological and methodological aspects.** Adviser: Carlos Frankl Sperber. Co-advisers: José Henrique Schoereder and Og Francisco Fonseca de Souza.

Ecological theory has proposed several mechanisms that might hold an effect on species diversity. Amongst so many possibilities, the aim of this thesis was to investigate crickets' biodiversity response to forest regeneration time and resource availability as well. To represent the different times of forest recovery, we sampled a chronosequence represented by several forest fragments in different regeneration times. On the first chapter of this thesis, we tested the response of crickets' species richness and composition against forest regeneration time. We evaluated percentage of canopy cover and litter depth as environmental variables. Species richness increased asymptotically with forest regeneration time and linearly with canopy cover and litter depth. Furthermore, when tested against forest recovery time, canopy cover had a positive linear relationship while litter depth had an asymptotical increase. Species composition was completely different among sampled forest fragments, with different regeneration times. This result highlights the importance of considering species composition in studies appraising response of species diversity to environmental disturbances. We found that even after species richness has attained a steady state, composition kept changing. Besides, the increase observed in species richness due to regeneration time and environmental variables may be caused by changes and amelioration in some resources and conditions required. This effect is thought to be even stronger for more susceptible species. Hence, crickets' diversity recovery encompasses an enhancement of niche complementarity together with changes in species composition. On the second chapter, we tested the hypothesis that the increase in resource availability should promote an increase on species richness, a change on species composition and a reduction on the species evenness of this community. Species richness did increase with resource availability, though the difference was only observed against negative control, without resource addition. Species composition changed and we observed an interaction among species identity and the resource availability (two levels: "no addition" *vs.* "addition"). Species evenness shrunk with resource availability. Although crickets are omnivorous, our results

enabled us to infer that diversity of these organisms is regulated by the resource availability. As reported on the literature, crickets depend on specific resources' sources, as sugars, to complement their dietary requirements. Resource addition may promote individual aggregation of rare species, shifting community structure and dwindling community evenness. Third and fourth chapters resulted from field observations and laboratory experiments. During data sampling, we observed that the pitfall traps we installed, using a standard cricket killing solution, proposed in 2003 did not captured some cricket species, even though they were abundant on forest floor and litter. Laboratory tests aiming the use of this data for molecular tests also revealed that the standard solution also allowed a rapid degradation of the DNA. Due to these drawbacks with this killing solution, we proposed its substitution for a new solution, the ethanol fuel. On the third chapter we tested the ability of ethanol fuel as a killing solution and its ability to preserve DNA. Using ethanol fuel as killing solution, we captured higher species richness and accumulated abundance in the same time and DNA was well preserved as well. Moreover, ethanol fuel is cheaper than commercial ethanol, logistically suitable, as it is easy to be found around field stations, sustainable and non-toxic. However, what is the reason why ethanol fuel captured more species and individuals? This question was answered in the fourth chapter. We tested that the higher capture rate is related with either higher attraction or diminished escape chance. Apart from not being attractive, in ethanol fuel individual sunk and died faster that the standard 2003 solution, reducing escape chance and explaining the higher sampling efficiency observed. Attractiveness would be a downside, as this characteristic could entangle occurrence of local organisms that felt on the trap with those occasional species that came from neighbor habitats attracted by the substance. Still, attractiveness would seriously impair comparisons with previous studies. Then, aside from taxonomic, anatomical and molecular studies, ethanol fuel can be used in ecological surveys, with few or any interference of the killing solution on the cricket sampling. In Brazil, after the publication of these studies, ethanol fuel has turned into the new standard solution used to sample crickets.

1 Introdução Geral

As atividades humanas estão promovendo ampla degradação ambiental, em escala e velocidade sem precedentes (Brooks et al., 2002). Por esse motivo, os cientistas estão concentrando esforços na investigação e determinação de fatores que afetam a diversidade de espécies, de modo a identificar os meios para assegurar a conservação da diversidade restante. Não tem faltado financiamentos para pesquisas que testem hipóteses mecanicistas e que avaliam correlações entre a diversidade de espécies e variáveis ambientais, em diversas escalas. Tem havido um grande esforço de pesquisa dedicado ao estudo da biodiversidade, especialmente no Estado de São Paulo (Joly et al., 2010, Programa Biota FAPESP). Esse esforço, recentemente, foi ampliado para todo o país, através do Programa SISBIOTA (Escobar, 2010). O Programa SISBIOTA, em particular, evidencia a necessidade de avaliar correlações entre a diversidade de espécies e variáveis ambientais, em diferentes escalas espaciais e temporais.

Embora os resultados de estudos de correlações não sejam conclusivos, estes podem gerar evidências de padrões de distribuição da diversidade, que podem ser testados em experimentos manipulativos. Para construir hipóteses razoáveis, é essencial baseá-las em previsões teóricas. A teoria ecológica propõe uma série de hipóteses e teorias que podem afetar a diversidade de espécies, dentre as quais, a teoria da sucessão ecológica (Clements, 1936) e da quantidade e diversidade de recursos (Tilman, 1982). Tais teorias ou hipóteses, entretanto, podem ser contrastadas com explicações mais parcimoniosas, como a teoria da amostragem passiva (Coleman, 1981). Esta teoria a medida que aumenta o número de indivíduos, aumenta a probabilidade de encontrar maior número de espécies. A resposta da comunidade de grilos a essas teorias constituíram o objeto central de estudo dessa tese.

A sucessão ecológica ou regeneração florestal ocorre em áreas que passaram por algum tipo de perturbação, seja em ambientes anteriormente desprovidos de vida (sucessão primária), ou ambientes anteriormente ocupados (sucessão secundária), que sofreram algum tipo de distúrbio natural ou antrópico (Begon et al., 2006). O cessamento desses distúrbios permitem que as plantas se estabeleçam de forma gradual, promovendo também a recolonização da fauna, prevenindo que espécies susceptíveis sejam extintas (Wright & Muller-Landau, 2006). Habitats em regeneração podem ser considerados refúgios para populações ameaçadas ou atuar como *stepping-stone*, facilitando o fluxo gênico entre habitats desconectados (Myers et al., 2000). Entretanto, a manutenção de espécies em florestas em regeneração depende diretamente da produtividade primária desta. Sem recursos e condições adequadas, muitas espécies não conseguem sobreviver. É por esse motivo que alterações na abundância, diversidade e na estrutura da comunidade são associadas a mudanças que ocorrem durante o processo de regeneração florestal. Espera-se, portanto, que a medida em que o tempo de regeneração aumenta, aumente a capacidade de suporte do ambiente, e este apresente maior densidade e diversidade.

No primeiro capítulo dessa tese avaliou-se a resposta da riqueza de espécies de grilos ao tempo de regeneração florestal. Testou-se a hipótese de que a riqueza de espécies aumentaria com o tempo de regeneração florestal. A hipótese foi baseada em Clements (1936), que prevê que a riqueza de espécies aumentava com o tempo de regeneração até atingir o clímax. Avaliou-se também como a riqueza de espécies responde a variáveis ambientais locais, como cobertura de dossel e profundidade de serrapilheira. Contrastou-se a teoria da regeneração florestal com a explicação mais parcimoniosa de que o efeito sobre a riqueza de espécies, nada mais é do que um efeito sobre o número de indivíduos (Coleman, 1981). Esse capítulo foi publicado, em 2012, no periódico *International Journal of Zoology*.

A disponibilidade de recurso é outro importante mecanismo que pode moldar a distribuição dos organismos no ecossistema (Tiegs et al., 2008; Lessard et al., 2011). A resposta da diversidade à disponibilidade de recursos pode ser representada por uma curva em forma de sino (Godfray & Lawton, 2001). Em ambientes com baixa disponibilidade de recursos deve ocorrer baixa diversidade, que pode ser explicada pela intensa competição intra- ou interespecífica (Ricklefs & Schluter, 1993). Diminuição na diversidade também pode ser observada quando há grande quantidade de recursos devido, principalmente, a competição intra- e interespecífica (Ricklefs & Schluter, 1993; Schmid, 2002) e/ou pela alta pressão exercidas por predadores (Araújo et al., 2007). Em faixas intermediárias de disponibilidade de recursos o ambiente pode suportar grande diversidade de organismos, devido ao aumento no número de indivíduos e com isso no número de espécies (Preston, 1962), ou por permitir maior coexistência entre as espécies (Godfray & Lawton, 2001). Esse padrão de curva em forma de sino, entretanto, ainda é controverso, especialmente porque a resposta dos organismos à disponibilidade de recursos é muito heterogênea (Mittelbach et al., 2001; Payne et al., 2005).

Seria de se esperar que grilos, que são considerados onívoros (Huber et al., 1989), não fossem afetados pela disponibilidade de recursos. Porém, embora os grilos apresentem uma dieta onívora-herbívora, esses organismos dependem de frutos, fungos e tecido animal para complementar sua dieta alimentar (Huber et al., 1989), além de açucares, que pode ser um recursos essencial. Além disso, os grilos podem depender de recursos como sítios de oviposição, territórios, água e profundidade da serrapilheira (McCluney & Date, 2008; Szinwelski et al., 2012), fatores que podem moldar usa distribuição espacial.Então como a disponibilidade de recursos regula a diversidade de grilos?

No segundo capítulo avaliou-se, através de experimento manipulativo, como a disponibilidade de recursos afeta a riqueza de espécies de grilos. Testou-se a hipótese de que o aumento na quantidade de recurso promoveria aumento na na riqueza de espécies devido ao afrouxamento das relações competitivas, alteraria a composição de espécies devido ao aparecimento de espécies raras, e reduziria a igualdade da comunidade, também chamada de equidade (Magurran, 2004). Esse capítulo está sendo preparado para ser submetido ao periódico *Organisms Diversity and Evolution*.

As metodologias de coletas utilizadas para estudar/entender os mecanismos determinantes da diversidade podem afetar o modo com que se estima a diversidade de um determinado ambiente. Isso porque, em geral, os cientistas não fazem um censo e sim uma estimativa da diversidade que pode estar distante ou próxima da verdade. Essa distância em relação ao mundo real está diretamente ligada aos métodos de amostragem e análise utilizados. Metodologias de coletas inadequadas ou insuficientes, podem, portanto, impedir que se detecte padrões sobre a riqueza de espécies, ou que se utilize os dados coletados para outros estudos.

A busca por técnicas adequadas de amostragem de Orthoptera tem sido aprimoradas ao longo do tempo. Inicialmente, utilizava-se a coleta manual desses organismos tanto para estudos taxonômicos quanto ecológicos (Otte & Alexander, 1983). Coletas manuais fornecem informações úteis ao pesquisador, como hábito e comportamento dos grilos, mas são de pouca utilidade para comparações ecológicas, devido à interferência do coletor (Southwood & Henderson, 2000). A utilização de *pitfall-traps* (Dahl, 1896) para a amostragem de grilos foi a alternativa encontrada para minimizar ou excluir o efeito do coletor em estudos ecológicos. Para capturar os grilos quando estes caem na armadilha, é preciso que esta contenha uma solução inseticida onde o animal se afogue (Sperber et al., 2003), pois caso contrário, esses organismos conseguem fugir facilmente. Antes de 2003, utilizava-se uma solução aqu-

osa, com um pouco de detergente para quebrar a tensão superficial da água. Essa solução, entretanto, além de degradar rapidamente os organismos coletados e seu DNA, possibilitava que muitos indivíduos escapassem da armadilha, prejudicando a amostragem. Em 2003, Sperber et al. propuseram a substituição dessa solução aquosa por um solução alcoólica, composta de 80% de álcool comercial, 10% de formol e 10% de glicerina. Essa técnica se mostrou mais eficaz que a anterior, pois além de capturar maior número de indivíduos e espécies, também conservava os indivíduos intactos por mais tempo. Além disso, essa solução não atrai, sendo eficaz para estudos ecológicos cujo objetivo é amostrar a fauna do local. Entretanto, com a utilização dos organismos coletados para outros estudos, de outras áreas da ciência, como por exemplo biologia molecular, essa solução mostrou-se ineficaz porque degrada o DNA rapidamente. Além disso, muitos grilos comuns na serrapilheira, como *Eneoptera* e *Gryllus*, que voam muito bem, ainda eram pouco amostrados com a utilização dessa solução. Pensando nessas duas ineficiências dessa solução, foi proposto a utilização de uma nova solução mortífera para a amostragem de grilos de serrapilheira.

O terceiro e quarto capítulos dessa tese foram resultados de observações de campo e testes laboratoriais. Em várias amostragens de grilos, observava-se que algumas espécies, embora abundantes na serrapilheira das florestas, não eram capturadas nas armadilhas que continham a solução matadora padrão, proposta em 2003, para amostragem de grilos. Além disso, com a aprovação do projeto *Biota de Orthoptera do Brasil* (CNPq-SISBIOTA 563360/2010-0), tornou-se necessária uma metodologia de campo que atendesse a todas as linhas do projeto, especialmente biologia molecular. Em testes laboratoriais, verificou-se a solução mortífera padrão degradava rapidamente o DNA, impossibilitando o uso dos organismos coletados para trabalhos que envolvessem biologia molecular.

No terceiro capítulo dessa tese, foi proposto a utilização do álcool combustível, sem diluição, como solução matadora para amostragem de grilos. Foi testado a eficiência do álcool combustível como solução matadora, comparando com a solução proposta por Sperber et al. (2003), e uma solução alternativa, composta de álcool comercial e glicerina, sem a adição de formol. Foi testado também a capacidade dessas três soluções de preservar adequadamente o DNA dos indivíduos capturados e por quanto tempo. Esse capítulo foi publicado, em 2012, no periódico internacional *Zookeys*.

O quarto capítulo resultou de uma pergunta gerada no capítulo anterior. Testou-se, através de experimento manipulativo, porque o álcool combustível captura maior número de indivíduos e espécies. Testou-se a hipótese de que a maior taxa de captura está relacionada a atração desses organismos pelo álcool combustível e/ou que o álcool combustível reduz a chance de fuga, devido principalmente, a sua baixa tensão superficial. Esse capítulo foi submetido ao periódico *Entomologia Experimentalis et Applicata*, e encontra-se em revisão.

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2 Capítulo um

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Research Article

Effects of Forest Regeneration on Crickets: Evaluating Environmental Drivers in a 300-Year Chronosequence

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We evaluated the relation of cricket species richness and composition with forest regeneration time, evaluating canopy and litter depth as environmental drivers. Effects of forest patch area, nearest distance to the 300-year patch, cricket abundance, sampling sufficiency, and nestedness were also evaluated. We collected 1174 individuals (five families, 19 species). Species richness increased asymptotically with regeneration time and linearly with canopy cover and litter depth. Canopy cover increased linearly, while litter depth increased asymptotically. Richness was not affected by patch area and nearest distance to the 300-year patch. Richness increased with cricket abundance, and this explanation could not be distinguished from regeneration time, evidencing collinearity of these two explanatory variables. Rarefaction curve slopes increased with regeneration time. Species composition differed among patches, with no nested pattern. We suggest that regeneration and consequent increases in canopy and litter promote recovery of cricket biodiversity, abundance, and changes in species composition. We conclude that the recovery of cricket diversity involves an increase along the spatial scale of complementarity, together with a change in species composition.

1. Introduction

Forest disturbances may range from simple alterations, such as light gap formation resulting from a toppled tree, to massive damage associated with large storms, hurricanes, fires, and human activities [1]. In tropical ecosystems, human activities—such as logging, mineral extraction, agriculture, and urbanization [2, 3]—are largely responsible for forest loss. These activities have caused losses in biodiversity [4] by reducing large areas of old-growth forest to small isolated forest patches. Forest patches are more affected by natural hazards than pristine, large forest areas [5] and are thus more susceptible to further reductions in diversity.

The abandonment of habitat patches, with the subsequent cessation of human activity, allows for forest regeneration and potential biodiversity recolonization [1, 6]. Forest landscapes are therefore often comprised of patches with different regeneration times [7–9].

Forest regeneration can reduce or eliminate threats to biodiversity [10] by provisioning suitable habitats for endangered species to prevent them from becoming extinct. Forest patches can function as habitat refuges, preserving threatened populations [11], and edge habitats can maintain both old-growth and secondary forest species [12]. Furthermore, forest patches may act as "stepping-stone" habitats that facilitate gene flow among otherwise disconnected forest patches [4]. However, the suitability of secondary forests for maintaining populations depends on the availability of adequate resources and conditions within the habitats of target species [13].

Changes in abundance, diversity, and species composition are commonly associated with succession because of the

environmental changes that occur during the regeneration process [14]. Several contradictory hypotheses have been proposed [14] to explain various patterns of diversity and species composition in succession gradients: (i) diversity should increase over succession time as the structural complexity of the ecosystem increases [15], or due to facilitation [16]; (ii) all species are present at the beginning of succession and several species may be eliminated by competition [17], resulting in decreased species richness during the succession process; (iii) because of intermediate disturbance effects, species diversity increases from early succession stages to a maximum in mid-succession and decreases during late succession [16, 18–20]; (iv) there is no general pattern of diversity during forest succession [21]; (v) given a uniform environment, with a fixed area, an increase in individuals leads to an increase in species [22].

In the case of litter crickets, the first hypothesis is possibly the most appropriate. Crickets respond to litter disturbance and trampling [23] and changes in environmental conditions, particularly humidity [24]. Given that early regeneration stages represent high-disturbance conditions—low humidity and low structural heterogeneity [25]—low cricket species richness is expected during such periods; therefore, higher richness is likely to be observed as the forest regenerates.

Our aim was to test if cricket species richness and composition responded to regeneration time and to evaluate potential local environmental drivers of species richness, that is, canopy and litter depth. We evaluated eventual landscape configuration effects, namely, forest patch area and nearest distance to the 300-year patch, and the eventual effects of cricket abundance on cricket species richness. Furthermore, we evaluated sampling sufficiency and evaluated if species composition differences could be explained by nestedness.

2. Methods

2.1. Study Region. The study was conducted in the Foz do Iguaçu municipality (25° 32′S, 54° 35′E, 195 m above sea level), Paraná State, in October 2008. Vegetation is composed of tropical semideciduous forest and ombrophilous mixed forest, within the Atlantic Rainforest biome [26]. The climate in this region can be categorized as humid subtropical mesothermal, with a mean annual temperature of 18–20°C and a mean annual rainfall of 1600 mm. The dry and rainy seasons range from April to June and October to January, respectively. Humidity is permanently high, seldom recorded below 80% even during the driest period [27].

At the time of this sampling, the canopy layer was already homogeneously closed, with most leaves completely developed. Therefore, the canopy layer was close to its maximum productivity, which is attained during the rainy season (N. Szinwelski, personal observations). During occasional observations in the dry season (May and June 2012), we did not observe strong canopy deciduousness.

2.2. Forest Disturbance History. We sampled a chronosequence of seven patches (Figure 1), ranging from zero to 300 years of regeneration (Table 1), from partial to total forest clearing. The patch with zero years of regeneration (Figure 1(a)) was totally cleared. The six-year patch was partially deforested (upper left corner, Figure 1(b)) and had suffered complete burning. The 15-year patch was almost entirely deforested, except for a narrow forest strip along the river which transects the patch longitudinally (Figure 1(c)). The 35- and 70-year patches suffered almost complete deforestation (Figures 1(d)-1(e)). The 130-year patch suffered partial deforestation. There is no recorded history of logging or human disturbance in the 300-year forest patch.

The patches of 0 to 70 years (Figures 1(a) to 1(e)) are presently private property; their ages were obtained from information provided by present owners and the descendants of former owners. The 130-year forest patch (Figure 1(f)), located in the Iguaçu River Basin on the western side of Iguaçu National Park [29, 30], was dated with information from the Paraguayan War that occurred between 1864 and 1870 [31]. During the war, the current site of the 130-year forest patch was deforested to build a road and to house troops, as reported by oral histories of local inhabitants. Presently, the 130-year patch is part of the Iguaçu National Park.

Although we assumed an age of 300 years for the oldest forest area (Figure 1(f), 300 years), this is probably an underestimation. The administration of the Iguaçu National Park considers the area, located in the Floriano River Basin, in the eastern region of Iguaçu National Park [29, 30], to be untouched wilderness (Marina Xavier and Apolônio Rodrigues, researchers at the Brazilian Institute for the Environment (Instituto Brasileiro do Meio Ambiente (IBAMA), personal observations). The Floriano River Basin is considered the only completely protected river basin in Southern and Southeastern Brazil [32] and was declared a world natural heritage site by UNESCO in 1986 [30].

Although presently the 130- and 300-year study areas belong to the same forest patch in Iguaçu National Park (Figure 1(f)), until 2002 these areas were separated by the Colono Road [30].

2.3. Testing the Assumption. To evaluate the effects of forest regeneration, we estimated regeneration using a continuous, rather than categorical (e.g., initial, intermediate, and late succession) approach. To achieve this, we used only the seven forest patches in the studied region for which precise knowledge of regeneration time was available. An increase in the number of sampled patches would only be possible if we included patches with inexact regeneration time data, which would jeopardize our approach.

At each forest patch, at least 200 m from the patch border, we placed 10 sets of pitfall traps parallel to each other at 15 m intervals, with each set consisting of a line of 5 traps 1 m apart. Each pitfall trap contained a solution of 80% ethanol, 10% formaldehyde, and 10% glycerin as a killing and preservative agent, as recommended by Sperber et al. [33]. The traps were maintained in the field for 48 hours, after which they were collected, and the crickets were

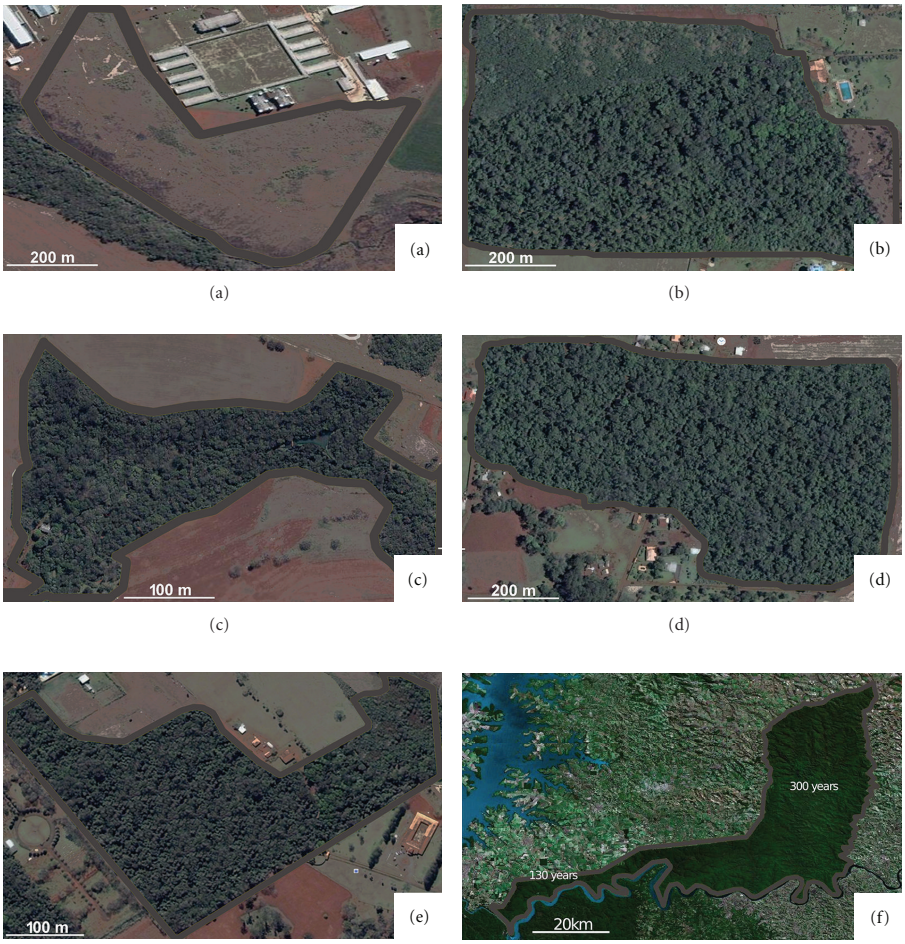

Figure 1: Study areas, with the following years of regeneration: (a) zero; (b) six; (c) fifteen, (d) thirty-five; (e) seventy; (f) 130 and 300 years (Iguaçu National Park). Source: [28]. For additional information, see Table 1.

Table 1: Characteristics of the sampled forest patches. Geographical coordinates correspond to the central point in each patch.

Regeneration time (years)	Geographical coordinate	Area (ha)	Distance to 300-year patch
0	25°28′05″–54°34′12″	21.29	25
6	25°34′19″–54°30′41″	44.25	10
15	25°27′51″–54°34′40″	6.35	25
35	35°35′02″–54°30′06″	36.09	8
70	25°33′03″–54°33′16″	6.66	15
130	25°37′54″–54°27′38″	35000	45
300	25°13′41″–53°44′57″	150000	0

sorted and stored in 80% ethanol. Voucher specimens were deposited in the Laboratory of Orthoptera, part of the *Museu Regional de Entomologia da Universidade Federal de Viçosa* (UFVB).

2.4. Potential Local Environmental Drivers. To evaluate potential environmental drivers of the cricket community, we measured litter and canopy structure. Litter depth was measured with a ruler at each trap. Mean litter depth was based on 50 samples per unit area.

To evaluate canopy cover, we took photographs at the intersection of each set of traps along the transect in each area, using a digital camera (CANON EOS 350-D Digital Rebel) with a fish-eye lens (Canon EF 15 mm f/2.8), positioned 1 m above ground level. The percentage of canopy cover was calculated using the program Gap Light Analyzer (GLA) [34]. For evaluation purposes photographs were converted into black and white, so that the amount of white pixels could be calculated (as a direct estimate of light penetration and an inverse estimate of cover) using GLA software. Canopy cover was calculated as the mean of the 10 samples from each area.

2.5. Landscape Configuration Effects. To evaluate if landscape configuration affected cricket species richness, we measured forest patch area and nearest distance to the 300-year patch using satellite images [28] and land title deed data provided by the land owners. We considered the distance to the 300-year patch as an estimate of species dispersal distance, because in addition to being the most preserved forest patch, it is also the largest continuous forest area in the region (135,000 ha + 50,000 ha of the 130-year patch, to which it is currently connected).

2.6. Data Analysis

2.6.1. Testing the Assumption. To test the assumption that cricket species richness increased with forest regeneration time, we adjusted generalized linear models (GLMs) with Poisson's errors, with accumulated species number per patch as response variable and regeneration time as an explanatory variable ($n = 7$, Figure 1). We used Chi-square (χ^2) test for Poisson's distributions and the F test when over- or under-dispersion was corrected, as recommended by Crawley [35] and Zuur et al. [36]. To evaluate the significance of the explanatory variable, we used stepwise backward model simplification, using the P value to exclude nonsignificant variables. Adjusted models were subjected to residual analyses, to evaluate the adequacy of the model. We detected evidence of nonlinearity that was not adequately modeled by including a quadratic term in a polynomial regression. We therefore adjusted nonlinear regression (*nls* procedure in *R*) with asymptotic models and evaluated the adequacy of the adjusted models by visual inspection of the predicted and observed values. Comparison of Akaike's information criterion (AIC) of the models was not available because the linear model presented overdispersion; therefore it did not provide this index.

2.6.2. Testing the Potential Local Environmental Drivers. To evaluate the potential local environmental drivers of cricket response to regeneration time, we tested the hypothesis that the variation in cricket species richness with regeneration time was driven by canopy cover and litter depth. We adjusted separate GLMs with cricket species richness and potential local environmental drivers as response variables. To avoid pseudoreplication, we considered the forest patches as our sampling unit ($n = 7$; Figure 1), using the mean values for litter depth and canopy cover per forest patch. For models with species richness as the response variable, we used Poisson's errors, and corrected for under- or overdispersion when necessary. For models with litter depth as the response variable, we used normal errors, since depth is a continuous variable. For models with canopy cover percentage as the response variable, we used binomial errors corrected for continuous data, since canopy cover is a proportion.

To evaluate the significance of the explanatory variable, we used stepwise backward model simplification, using the P value to exclude nonsignificant variables. Adjusted models were subjected to residual analyses, to evaluate model adequacy. If an environmental variable was an effective driver of the response of richness to regeneration time, we expected that richness would be affected by this variable and that the variable would correlate to regeneration time.

We detected evidence for nonlinearity in the relationship of litter depth with regeneration time. This could not be adequately modeled by including a quadratic term in a polynomial regression, so we adjusted nonlinear regression (*nls* procedure in *R*) with asymptotic models and evaluated the adequacy of the adjusted models by visual inspection of the predicted and observed values. We used AIC values to choose the most adequate model.

2.6.3. Testing Landscape Configuration Effects. To evaluate if landscape configuration explained the response of cricket species richness to forest regeneration time, we adjusted GLMs with species richness as the response variable, regeneration time as the explanatory variable, and patch area and nearest distance to the 300-year patch as covariables, adjusted logistic multiple regression with Poisson's errors, and adjusted for under- or overdispersion as necessary. The complete model to evaluate the effects of landscape configuration included all interaction terms. To evaluate the significance of the explanatory variable, we used stepwise backward model simplification, using the P value to exclude nonsignificant variables. Adjusted models were subjected to residual analyses to evaluate model adequacy.

2.6.4. Testing for the Effects of Cricket Abundance on Cricket Species Richness. To evaluate if cricket abundance would explain cricket species richness, we adjusted GLMs with cricket species richness per patch as the response variable ($n = 7$), regeneration time as the explanatory variable, and cricket abundance as the covariable, adjusted logistic multiple regression with Poisson's errors, and adjusted for under- or overdispersion as necessary. The complete model to evaluate the effects of cricket abundance on the studied

relationships included all interaction terms. To evaluate the significance of the explanatory variable, we used stepwise backward model simplification, using the P value to exclude nonsignificant variables. Adjusted models were subjected to residual analyses to evaluate model adequacy.

Cricket abundance was estimated by the total number of individuals captured in the 50 traps of each studied patch. Eventual significance of abundance effects on species richness was interpreted as passive sampling [37], where patches with more individuals presented larger species richness.

All univariate analyses were done within the *R* environment [38].

2.6.5. Testing for Sampling Sufficiency. To evaluate sampling sufficiency for estimating the species richness of each patch, we used individual-based rarefaction analysis [39], comparing species richness accumulation curves among patches by visual assessment of overlapping 95% confidence intervals. Rarefaction analysis was done in EstimateS 7.5 [40].

2.6.6. Testing for Effects of Regeneration Time on Cricket Species Composition. To evaluate if species composition differed among forest patches, we considered each group of five pitfall traps as our sampling unit ($n = 70$), to evaluate if the variation within patches was larger than the variation among patches. We assumed that species composition differed among patches when sampling units of a particular patch were more similar to each other than to those from different forest patches. To analyze the similarity among samples, we used nonmetric multidimensional scaling (NMDS), running 10,000 permutations and using the Bray-Curtis distance to explore differences in community structure across the patches.

We used the stress value to assess the robustness of the NMDS solution, as stress values above 0.2 indicate plots that may be unreliable [41]. Analysis of similarity (ANOSIM) was used to test if there were significant differences in multivariate community structure among forest patches. The null hypothesis was that there would be no differences among forest patches. ANOSIM is a nonparametric permutation test for similarity matrices analogous to analysis of variance (ANOVA) [41]. We used similarity percentage analysis (SIMPER) to evaluate which species are more relevant to group forming. All multivariate analyses were undertaken using PAST software [42].

2.6.7. Nestedness Analyses. To evaluate if species composition differences could be explained by nestedness, that is, if cricket species in forest patches with lower species richness were a subset of the species present in higher-richness sites [43, 44], we measured the degree of nestedness of the cricket assemblages from the seven forest patches using the "*vegan*" library [45] of the *R* environment [38]. We calculated the NODF (nestedness metric based on overlap and decreasing fill) statistics [46], running 10,000 simulations using the "$r1$" method, which uses both row and column constraints as recommended by Ulrich et al. [44]. The NODF statistics vary from 0 to 100, with 100 representing maximum nestedness [47].

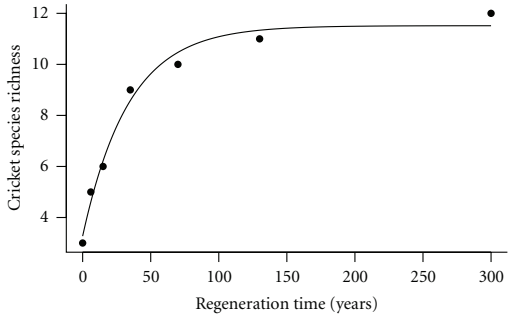

Figure 2: Response of cricket species richness to regeneration time. Species richness increased asymptotically up to 130 years of regeneration. Nonlinear regression with Gaussian errors: $y = 11.293 - 8.081^{(-0.003*x)}$; $F_{2,4} = 16.16$; $P = 0.012$.

3. Results

3.1. Cricket Fauna. We collected 1174 individuals belonging to five families and 19 species. The richest and most abundant family was Phalangopsidae (12 species: 983 individuals), followed by Trigoniidae (two species: 107 individuals), Eneopteridae (two species: nine individuals), Gryllidae (two species: 70 individuals), and Mogoplistidae, which had only one species and five individuals (Table 2). Crickets of the Gryllidae family occurred only in areas with zero years of regeneration (open habitat) and were absent from the remaining areas, while five species of Phalangopsidae were exclusive to older forests (Table 2).

3.2. Testing the Assumption. Using linear regression, we detected that cricket species richness increased with forest regeneration time (overdispersion; $F_{1,5} = 22.37$; $P = 0.005$), but there was strong evidence of nonlinear relation. The relationship between species richness and regeneration time was adequately modeled by the following asymptotic equation:

$$y = 11.293 - 8.081^{(-0.003*x)}. \quad (1)$$

Therefore, cricket species richness increased asymptotically with regeneration time until stabilizing at 130 years of regeneration (nonlinear regression; Figure 2).

3.3. Local Environmental Drivers. Cricket species richness increased with percentage of canopy cover ($\chi^2 = 3.97$; $P = 0.046$; Figure 3) and litter depth ($\chi^2 = 8.15$; $P = 0.004$; Figure 4).

Canopy cover increased with forest regeneration time ($F_{1,4} = 54.24$; $P = 0.018$; Figure 5). Litter depth was not linearly related to regeneration time ($F_{1,5} = 5.30$; $P = 0.06$), but there was a strikingly nonlinear relationship. When using nonlinear regression to adjust an asymptotic model, the relationship between litter depth and regeneration time was

Table 2: Cricket taxa, number of individuals per forest patch, and taxa contribution to species composition groups forming in SIMPER analysis (taxon alone (A), percent value (%), and taxon order (B)). Taxa were ordered according to contribution (B). Taxa not assigned to described species or genus received number codes. All unidentified crickets belong to taxa that had not been previously collected and are therefore new to science.

Taxons	Forest patches years								Taxa contribution		
	0	6	15	35	70	130	300	Total	A	%	B
Ectecous sp.1	—	85	33	34	157	147	194	650	32.82	44.78	1
Phoremia sp.1	—	—	—	85	5	5	10	105	8.43	56.28	2
Gryllus assimilis	49	—	—	—	—	—	—	49	6.86	65.64	3
Lerneca sp.1	6	45	30	—	23	—	—	104	6.36	74.32	4
Laranda sp.1	—	10	16	27	10	12	4	79	4.82	80.9	5
Vanzoliniella sp.1	—	9	24	23	8	—	—	64	4.19	86.61	6
Aracamby sp.1	—	—	—	3	15	8	10	36	2.36	89.83	7
Aracamby sp.2	—	—	—	—	—	14	17	31	2.33	93.02	8
Miogryllus sp.1	5	16	—	—	—	—	—	21	1.89	95.6	9
Adelosgryllus rubricephalus	—	—	2	2	1	1	1	7	0.62	96.45	10
Eneoptera surinamensis	—	—	5	—	—	—	—	5	0.61	97.29	11
Mogoplistidae Genus 3 sp.1	—	—	—	1	1	2	1	5	0.47	97.93	12
Phalangopsidae Genus 1 sp.1	—	—	—	—	—	3	1	4	0.39	98.46	13
Tafalisca sp.1	—	—	—	2	1	—	1	4	0.34	98.94	14
Phalangopsidae Genus 2 sp.2	—	—	—	—	—	1	2	3	0.24	99.27	15
Eidmanacris tridentata	—	—	—	—	—	1	1	2	0.17	99.5	16
Endecous sp.1	—	—	—	—	—	1	1	2	0.16	99.73	17
Zucchiella sp.1	—	—	—	—	2	—	—	2	0.13	99.91	18
Eidmanacris bidentata	—	—	—	1	—	—	—	1	0.06	100	19
Individuals	60	165	110	178	223	195	243	**1174**	—	—	—
Species	3	5	6	9	10	11	12	**19**	—	—	—

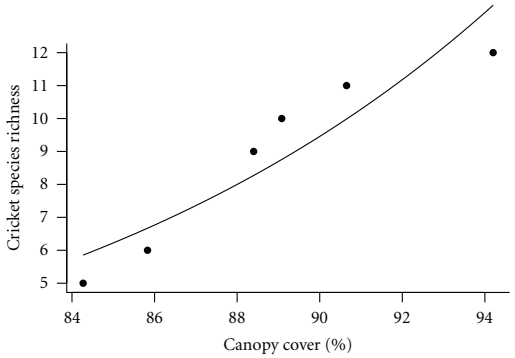

Figure 3: Response of cricket species richness to canopy cover. Species richness increased linearly with canopy cover. Linear regression with Poisson's errors: $y = e^{(-5.285+0.083*x)}$; $\chi^2 = 3.97$; $P = 0.046$.

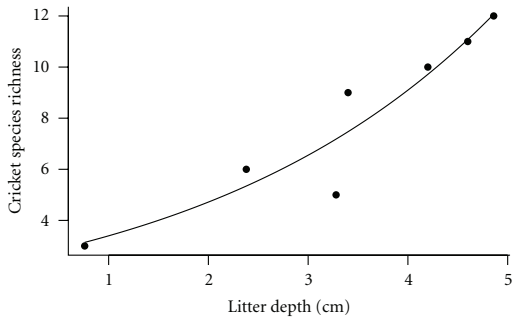

Figure 4: Response of cricket species richness to litter depth. Species richness increased linearly with liter depth. Linear regression with Poisson's errors: $y = e^{(0.894+0.328*x)}$; $\chi^2 = 8.15$; $P = 0.004$.

adequately modeled ($F_{2,4} = 8.78$; $P = 0.034$; Figure 6) by the following equation:

$$y = e^{(0.894+0.328*x)}. \quad (2)$$

3.4. Landscape Configuration Effects. Neither patch area nor nearest distance to the 300-year patch had any effect on cricket species richness ($\chi^2 = 3.24$; $P = 0.07$ and $\chi^2 = 0.25$; $P = 0.61$, resp.).

3.5. Effects of Cricket Abundance on Cricket Species Richness. There was no interaction effect of patch regeneration time with cricket abundance ($F_{1,4} = 4.06$; $P = 0.13$). The deletion of both cricket abundance and regeneration time was nonsignificant when compared to a model maintaining one of these explanatory variables (Y abundance + time

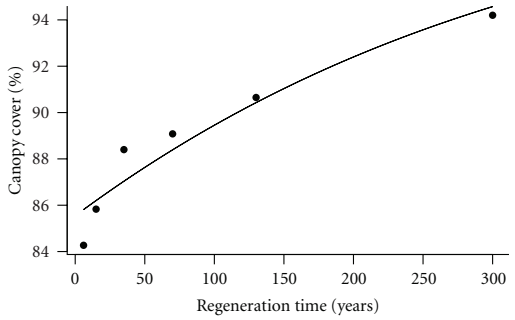

Figure 5: Response of canopy cover to regeneration time. Canopy cover increased linearly with regeneration time. Linear regression with binomial errors: $y = 100 * e^{(1.778+0.003*x)}/1+e^{(1.778+0.003*x)}$; $F_{1,4} = 54.24$; $P = 0.018$.

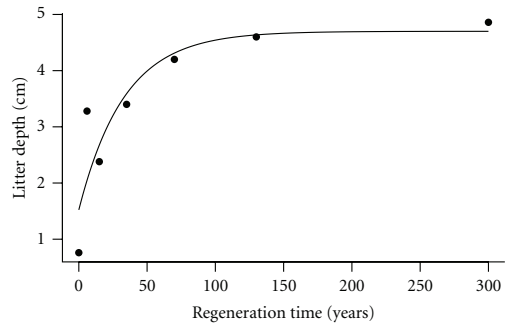

Figure 6: Response of litter depth to regeneration time. Litter depth increased asymptotically up to 130 years of regeneration. Nonlinear regression with Gaussian errors: $y = e^{(0.894+0.328*x)}$; $F_{2,4} = 8.78$; $P = 0.034$.

versus Y abundance; $F_{1,5} = 6.92$ $P = 0.068$; Y abundance + time *versus* Y time $F_{1,4} = 0.11$; $P = 0.75$). When compared to the null model, however, both explanatory variables significantly affected cricket species richness (Y abundance *versus* $Y1$; $F_{1,5} = 5.52$; $P = 0.045$ and Y time *versus* $Y1$; $F_{1,5} = 22.37$; $P = 0.005$). Therefore, cricket species richness per patch could be explained both by regeneration time and cricket abundance.

3.6. Sampling Sufficiency. Although we detected no statistical difference in rarefaction curves among forest patches, the slopes of the rarefaction curves increased with regeneration time (Figure 7). The bias of the estimated species richness increased, in correlation with the regeneration time. In the most recent forest patches (zero to 15 years of regeneration), species richness was fully sampled, while the rarefaction curves in all remaining, older, patches showed that we did not reach the actual species richness. Therefore, the rarefaction curves reinforce the pattern of increasing species richness with regeneration time.

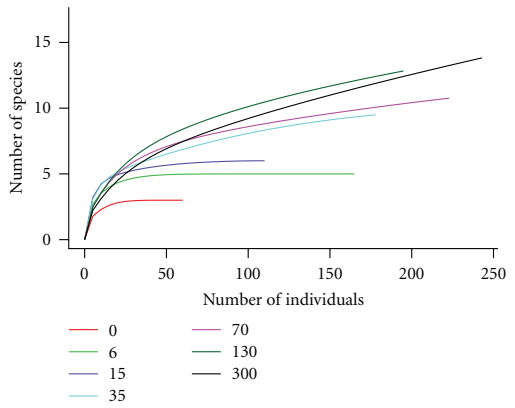

Figure 7: Individual-based species rarefaction curves for crickets communities within different forest patches. All 95% confidence intervals (CI) overlapped, showing that there was no significant difference between forests patches. We removed the dotted lines that represent CI, so as to allow visualization of trends.

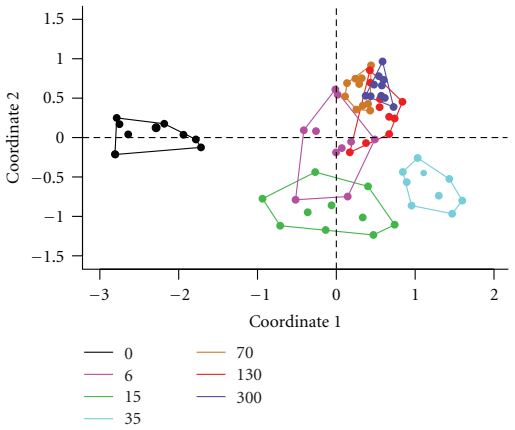

Figure 8: Plot of nonmetric multidimensional scaling (NMDS) ordination, showing difference between areas: stress 0.1401; $P < 0.001$. Colors correspond to regeneration time, varying from 0 to 300 years.

3.7. Effects of Regeneration Time on Cricket Species Composition. Species composition was different among all forest patches (Stress 0.1401; $P < 0.001$; Figure 8), with ANOSIM indicating complete separation among patches ($R = 0.75$; P (same) < 0.0001; Bonferroni P values for each patch combination < 0.03; Table 3).

The SIMPER (Table 2: taxa contribution) showed that *Ectecous* sp.1 and *Phoremia* sp.1 were the two most relevant species for group forming in the species composition NMDS analysis, with 45% and 56% cumulative contribution, sequentially.

Table 3: Analysis of similarity (ANOSIM) results, showing, Bonferroni-corrected P values for the null hypotheses that forest patch species composition is the same for each patch combination. Permutation number = 10,000; mean rank within = 419.6; mean rank between = 1326; $R = 0.7509$; overall P (same) < 0.0001; distance measure: Bray-Curtis.

Forest patch	Forest patch (regeneration time)						
(regeneration time)	0	6	15	35	70	130	300
0	—	0	0	0	0	0	0
6	0	—	0.0007	0.0001	0	0	0
15	0	0.0007	—	0	0	0	0
35	0	0.0001	0	—	0.0001	0	0
70	0	0	0	0.0001	—	0.0004	0.0039
130	0	0	0	0	0.0004	—	0.0244
300	0	0	0	0	0.0039	0.0244	—

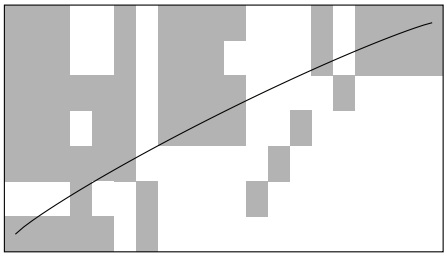

Figure 9: Presence (gray) or absence (white) of the 19 species (columns) in each of the seven forest patches (rows). For nested pattern, all species should appear above the curve. The result shows that species composition was not nested.

3.8. Nestedness Analyses. Species composition showed no nested pattern (NODF = 51.72; $P = 0.84$; Figure 9).

4. Discussion

4.1. Cricket Fauna. The exclusiveness of the Gryllidae family to open habitat coincides with previous observations [48] that this family is typical of open areas, in contrast to Phalangopsidae and Trigoniidae, which are characteristic of forest habitat. Open areas facilitate flight and allow sound to spread easily [49], leading to a predominance of winged species with well-developed posterior wings, which are responsible for flight [50]. Among the Gryllidae, most species had well-developed hindwings and stridulatory apparatus for acoustic communication [51]. This may explain why Gryllidae were restricted to the open area.

Sound propagation is limited in forest habitats, which may represent a selective pressure against acoustic communication [49, 52]. In forested areas, apterous species and those without posterior wings predominate, particularly in the case of litter crickets (C.F. Sperber, personal observations). Such species are unable to fly [53]. The loss of forewings implies the loss of stridulatory capacity. As a probable alternative form of communication, many litter cricket species have secretory external glands used in pre- and postcopulatory behavior. All of the Phalangopsidae that we collected lacked posterior wings, with the exception of *Lerneca* sp.1 (Grylloidea: Phalangopsidae).

Lerneca sp.1 presents developed posterior wings, similar to those of *Eneoptera surinamensis* (Grylloidea: Eneopteridae). Both species are good fliers and may be especially well adapted to dispersion. Although we collected *E. surinamensis* in only one area, this species is common in disturbed forest habitats [54].

Forest Phalangopsidae generally have slender, poorly chitinized bodies, which makes them more prone to desiccation and therefore dependent on humid conditions. This may explain their high abundance in regenerated forests. In contrast to the slender body of forest Phalangopsidae, the body of *Lerneca* is more robust and chitinized, making this taxon less dependent on humid conditions. Similarly, *E. surinamensis* also has a robust, strongly chitinized body and is not dependent on high humidity. This species probably absorbs water for metabolism from its diet, and its phenology is synchronized to seasonal water availability, remaining as nymphs (which are vulnerable to desiccation) during the rainy season and developing into adults in the dry season [55]. Similar adaptations may occur in *Lerneca* sp.1. The above characteristics explain why these two species are commonly collected in less regenerated forests.

The Phalangopsidae genera *Eidmanacris*, *Endecous,* and *Aracamby* are usually associated with less disturbed forests, being dependent on high humidity in the soil, shelter in armadillo holes, tree holes, or gaps formed by fallen logs [56]. *Phoremia* and *Zucchiella* (Trigoniidae) are recorded as associated with less disturbed forests [57] and use litter for displacement and sheltering [23].

The predominance of the Phalangopsidae species *Ectecous* sp.1, in relation to the Trigonidiidae species *Phoremia* sp.1 in regenerated forests (Table 2), contrasts with findings from other Atlantic Rainforest patches, where Trigonidiidae predominate [23]. This may be a result of topographical differences between the two studies: the areas studied here occur in flat topography, whereas *Phoremia* predominates in areas with a more pronounced topography, particularly hilly domains [58]. Another factor explaining the contrasting results of these studies is that the size of the forest patches

studied differed: while the size of the patches in this study varied from seven to 150 thousand hectares, forest patches where *Phoremia* predominates were all less than 350 ha [23]. Smaller areas are more susceptible to abiotic disturbances, such as edge effects [11, 59], and anthropogenic disturbances, such as selective logging [60]. If this is the case, then the predominance of *Ectecous* in Atlantic Rainforest litter could be regarded as an indicator of the degree of forest preservation.

4.2. Species Richness Response to Regeneration. The asymptotic response of cricket species richness to regeneration time (Figure 2) suggests that species accumulation occurs in two distinct phases. Species richness increases up to *ca.* 130 years of regeneration, when a local limit may be reached. However, we must take the asymptotic stabilization of species richness with regeneration time with caution, since the bias of the estimated species richness also increased with regeneration time, as depicted by the increasing slope of the rarefaction curves with regeneration time (Figure 7). At the spatial scale sampled here, however, our results show a trend of local species richness stabilizing with regeneration time, contrasting with a continuous change in species composition (Figure 8).

The asymptotic response of cricket species richness to forest regeneration could be interpreted as "how much is enough?" [61]; that is, a regeneration period of 130 years would be enough to restore original species richness. However, the continuity of the directional change in species composition may be regarded as evidence that this interpretation is incorrect. Although species richness did not change from 130 to 300 years of regeneration, species composition continued to change.

The asymptotic accumulation of cricket species differs from the patterns proposed in the literature. The observed response may be a subtle divergence from the constant increase expected by Clements [15]. On the other hand, the asymptotic response could correspond to the initial portion of the humpback pattern expected by intermediate disturbance [18]. Larger time spans would highlight the decreasing portion of the humpback pattern. Rosenzweig [62] already suggested that such partial gradient responses to explain contradictory patterns of increase and decrease of richness with succession.

Our chronosequence is, however, old enough to test whether further changes occurred over a longer time period. Our highest regeneration time was of at least 300 years. Any disturbance in this area would have been restricted to forest use by Amerindians, prior to European colonization of Brazil. Estimates of human population size at the time of first European contact range from 1 to 5 million, but the indigenous population has now declined to about 185,000 [63]. Moreover, according to the present knowledge, forest use and disturbance by Amerindians would have been spatially and temporary restricted [64]. Amerindians generally built in natural clearings, with selective logging and no pruning of roots [65]. We therefore believe that disturbances caused by Amerindians were spatially and temporarily restricted, and that the eventual effects of such disturbances upon forest litter communities would not persist until today.

4.3. Local Environmental Drivers. The mechanisms involved in the increasing levels of species richness include canopy cover and litter accumulation. However, the coincidence of the asymptotic litter response curve to regeneration (Figure 6) suggests that this environmental variable is fundamental to determining cricket species richness. The limit to species accumulation suggests that there is some kind of saturation point, mediated through competitive or other biotic interactions [10]. Litter depth could possibly correlate to shelter availability. Shelter within litter could provide both enemy protection [66] and favorable humidity conditions [24]. Species saturation could, therefore, be determined by bottom-up as well as top-down control mechanisms [67, 68]. If this is the case, litter cricket communities of old tropical forests might be saturated, even though competition for food is not apparent: crickets are omnivores, thus probably generalists; therefore food resources are probably not limiting. Shelter from natural enemies or suitable oviposition sites with more favorable environmental conditions may be limiting factors for litter crickets. Thus it is possible that crickets compete for these resources, creating a limit to species richness.

4.4. Environmental Drivers: Canopy Cover. The increase of canopy cover with regeneration time (Figure 5) leads to lower temperature variability and lower evaporation of soil water [66]. High temperature variation—typical of early succession stages [69]—can exceed insect thermoregulatory capacities, affecting development and survival [70]. Furthermore, variations in temperature can induce diapause in insects [71], resulting in a decreased metabolic rate [72] and compromised immune response [73]—which ultimately affects locomotion and reproduction [74, 75]. Increased canopy cover may therefore represent an increase in cricket habitat suitability [76], driving the observed increase in species richness (Figure 3).

Humidity affects reproduction in insects [77]. Since the reproductive rate of crickets may be reduced during low humidity conditions [24], it can be expected that a higher reproductive rate would be achieved in environments with greater canopy cover. Humidity can also affect insect locomotion, since it influences soil adhesiveness [74]. Litter crickets move by means of walking and jumping, and locomotion efficiency can also impact mating success and predation avoidance. High humidity may increase fungus development [78], which may reduce food palatability and facilitate the growth of toxin-producing entomopatogenic fungi [79] that can be lethal to insects (but see Elliot et al. [73]). Excessively high humidity may therefore be harmful to litter crickets.

Canopy cover can be correlated to the production of foods, such as fruits that are a common resource for litter crickets. Canopy cover can also be correlated to increased habitat structural complexity [80] resulting in increased litter depth. Litter may provide food resources [81, 82],

and a deeper leaf-litter layer could also provide a refuge for crickets to maintain humidity during the dry season; thus buffering population declines during such periods [83]. Litter is also important for the provision of nesting sites, especially for species that oviposit directly into the soil or litter components [84].

4.5. Environmental Drivers: Litter Depth. Litter depth responded asymptotically to regeneration time, stabilizing at 130 years (Figure 6), which converges with the observed response of species richness (Figure 2). We suggest that this parameter is the strongest environmental driver of cricket species richness. The stabilization of litter depth with increasing canopy cover may result from an increase in decomposition rate in old-growth forest [85]. High production of leaf litter thus corresponds with a high rate of decomposition.

4.6. Effects of Cricket Abundance on Cricket Species Richness. Our statistical results were inconclusive between choosing regeneration time or cricket abundance to explain species richness per patch. This doubt characterizes collinear explanatory variables [35, 36]. Collinearity occurs when explanatory variables covary in the field, with both variables contributing to the observed pattern. Therefore, both cricket species richness and abundance increased with forest regeneration time. One effect cannot be discussed separately from the other. We interpret these correlations as evidence of increasing habitat quality for crickets during forest regeneration.

4.7. Sampling Sufficiency. For the older (35 years or more) forest patches, the rarefaction curves suggest that the cricket species richness was undersampled, since there was no distinguished stabilization in the species accumulation curves (Figure 7). Although intensive sampling in the most preserved patch (300 years), done for taxonomy purposes (Francisco A. G. de Mello and Pedro G. Dias, personal communication), resulted in 25 cricket species (compared to 19 found here); thirteen of these cricket species live in tree trunks, shrubs, and canopy (Pedro G. Dias, personal communication) and are rarely caught in pitfall traps. All species found in the litter during that taxonomic study were also sampled here. Therefore, if there are undetected litter cricket species in the older forest patches, they must be very rare.

The increase in the slopes of the rarefaction curves with regeneration time (Figure 7) indicates an increase in the bias of the estimated species richness with forest regeneration, evidencing an increase in the spatial scale at which species richness is detected. In the most recent forest patches (zero to 15 years of regeneration), species richness was fully sampled, while the rarefaction curves in all remaining, older, patches showed that we did not reach the actual species richness. Therefore, the rarefaction curves reinforce the pattern of species richness increasing with regeneration time.

Our results suggest an apparent saturation of cricket species richness at the sampled spatial scale as well as an increasing complementarity (*sensu* Colwell and Coddington [86]) of cricket species composition within older forest patches. This may result from an increase in regional species richness, unveiling long-term evolutionary processes. Older forest patches may harbor a larger species pool, which could be traced back to the evolutionary history of the original forest habitat.

4.8. Effects of Regeneration Time on Cricket Species Composition. Although regeneration led to changes in species composition that were coincident with an increase in species richness, composition changes could not be assigned to nestedness; that is, species composition in lower-richness patches was not a subset of species composition in the higher-richness patches (Figure 9). This, along with the differences in composition detected in the NMDS, suggests a directional change in species composition. This coincides with classic definitions of ecological succession [87]. Our results indicate that there may be a directional replacement of species, driven by ecological succession.

4.9. Concluding Remarks. Our results highlight the importance of considering species composition when evaluating biodiversity changes after a disturbance. While the increase in species richness stopped after *ca.* 130 years of forest regeneration, species composition continued changing. The regeneration that we observed may be restricted to regions where there is a sufficiently large and well-preserved pool of late-succession species that constitute a source of colonizers for regenerating areas. Environmental drivers of biodiversity regeneration probably involve changes in both resource availability and favorable conditions. We believe that the same processes may drive biodiversity regeneration of other organisms, which share a strong dependence on local habitat. A general implication for conservation is that the evaluation of biodiversity recovery necessitates the evaluation of both diversity and species composition responses. Studies that consider only species richness may generate misleading conclusions.

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3 Capítulo dois

3.1 Resource addition improves cricket diversity?

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Resource addition improves cricket diversity?

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Abstract This study evaluates if resource availability drives litter cricket diversity. We aimed to evaluate the hypotheses that resource addition (i) increase species richness, through competitive release, allowing (ii) rare species to increase their populations, and, thus, (iii) reducing community evenness. To evaluate our hypotheses, we experimentally added sugarcane syrup in 6 levels, and evaluated species richness, species composition and evenness, using GLM and GLMM. Cricket species richness was higher when resource was added, compared to "no addition", but resource addition quantity did not affect species richness, so that resource addition was amalgamated in two levels ("no addition" and "addition"). The abundance of all cricket species captured in the "no addition" plots was not reduced by resource addition. The less abundant species in the "no addition" plots, *Phoremia zefai*, increased its abundance three times. Eleven cricket species were exclusively captured when resource was added. Therefore, we found evidence that resource availability is a driver of cricket diversity. Resource addition diminished community evenness and altered community composition. Sugarcane syrup addition promoted aggregation of individuals pertaining to rare species, that are present in the habitat, but whose density is low. This aggregation lead to an increase in the observed species richness, and altered community structure. The reduction in evenness, promoted by resource addition, was due to the increase in observed species richness. Our results are evidence that 12 of the 14 cricket species present in forest litter are maintained at low densities by resource scarcity. When resource availability is experimentally increased, species richness increases due to behavioral displacement, changing community structure.

Keywords Sugarcane · community structure · community evenness · behavioral displacement · Iguaçu National Park · manipulative experiment

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Introduction

Ecological theory predicts several alternative mechanisms that may drive diversity. It became increasingly clear that diversity drivers span multiple spatial and temporal scales (Ricklefs and Schluter, 1993; Leibold et al, 2004; Ricklefs, 2004). In regional scale, diversity can be determinated by dispersion and colonization events (Leibold et al, 2004; Chase and Bengtsson, 2010), obscuring local determination (Fukami, 2010). In local scale, ecological interactions may determine a limit to diversity imposed by enemy free space (Vanschoenwinkel et al, 2010) or competition for limited resources (Tilman and Pacala, 2003).

Resource availability is an important driver that can shape organisms distributions and ecosystem processes (Tiegs et al, 2008; Lessard et al, 2011). The response of diversity to resource availability can be represented by a hump-shaped curve (Godfray and Lawton, 2001). Environments with low resource availability can present low diversity, and it can be explain by extreme competition (Tilman and Pacala, 2003). At intermediate resource availability, the environment can support a greater diversity of organisms, due to increase in individuals number, hence increase diversity (Preston, 1962), or permitting species coexistence, without fight for resource (Godfray and Lawton, 2001). Decrease on diversity can be observed when high resource is available, due to intense intra- or interespecific competition (Schmid, 2002), and/or higher pressure of predation (Araújo et al, 2007), doing some species dominant in the environment. However, there were controversy about the hump-shaped curve because organisms response are quite variable, so that patterns found in nature are often heterogeneous (Mittelbach et al, 2001; Payne et al, 2005).

To date, little is known about the resources limiting of litter crickets. Litter crickets are recognized as omnivores, with a primarily herbivorous diet (Huber et al, 1989). Thereby, it is difficult to determine with resources are limiting for crickets and hence regulate cricket density and diversity. Oviposition sites, territories, water (McCluney and Date, 2008) and litter depth (Szinwelski et al, 2012) are resources limiting for many cricket species. Additionally, although cricket presented a omnivores-herbivorous diet, food can be limiting for crickets, specially because its depends on fruits (sugar), fungi and animal tissue to supplement its diet (Walker and Sakai, 1989). So, in a community of litter crickets, how resource availability drives cricket diversity?

In this study, we used manipulative experimental to evaluate if resource availability drives cricket diversity. We expect that resource addition will (i) increase species richness, through competitive release, allowing (ii) rare species to increase their populations, and, thus, (iii) reducing community evenness.

Material and methods

Study site

The sampling was done in old-growth Atlantic forest, in the Iguaçu National Park (25°32'52"S – 54°35'16"W), Foz do Iguaçu, PR, Brazil, during January 2010. The Iguaçu National Park have 185.000 ha of area and was declared a world natural heritage site by UNESCO in 1986 (Ortiz, 2010). Vegetation

is composed of tropical semideciduous forest and ombrophilous mixed forest, within the Atlantic rainforest biome (Rizzini, 1997). The climate in this region can be categorized as humid subtropical mesothermal, with a mean annual temperature of 18 – 20°C and a mean annual rainfall of 1600mm. The dry and rainy seasons range from April to June and October to January, respectively.

Experimental design

We did six parallel transects of 180m, starting at a distance of 5km from the forest edge, and 1000m distant from each other. At each transect we placed six sets of five pitfall traps ("A" to "E"), 30m distant from each other. In each set, the traps were placed perpendicularly to the transect, 2m apart from one another. Traps were polyethylene vials, 20cm in diameter and 22cm deep, filled with 500ml of alcohol + formaldehyde + glycerin as killing solution (Sperber et al, 2003). Traps were left in the field for 48h. Therefore we had a total sampling effort of 180 traps, aggregated in 36 sets (n=36).

Treatment consisted in hand-spraying 6 times sugarcane syrup (food resource), on the litter surrounding the pitfall trap. Previously, Szinwelski et al. (unpublished) showed that sugarcane syrup odor is an efficient bait for litter crickets, increasing the number of captured species and individuals in pitfall traps. Spraying was conducted on none ("no addition") to five ("one to five added") pitfall traps of each set of five pitfall traps, immediately after the traps were buried into the soil. The experiment was completely randomized, so that treatment level (none to five) and which pitfall within the set ("A" to "E") received the treatment, were chosen randomly. Therefore we had 6 treatment levels, varying from none to five pitfall traps receiving sugarcane syrup spraying. Each set of five-pitfall traps were considered our sampling unit (n=36).

Specimens were identified by Dr. Carina M. Mews, following the classification of Desutter (1987, 1988). Voucher specimens were stored in alcohol 80% and deposited in the Laboratory of Orthoptera, affiliated to the *Museu Regional de Entomologia da Universidade Federal de Viçosa* (UFVB).

Data analysis

To test if resource addition increased species richness, we adjusted generalized linear models (GLM) with number of species per set of five pitfall traps as response variable (n=36), and quantity of resource addition, as explanatory variable. To test if there was a linear response to resource addition quantity, we adjusted linear regression to the whole set of samples, with resource addition quantity as continuous variable. We compared this model to a model excluding the "no addition" samples, so as to evaluated if resource addition quantity *per se* affect species richness.

To evaluate if there was non-linear response to resource addition quantity, we used one-way analysis of variance (ANOVA), considering resource addition, as the explanatory factor, with six levels. Significance of level effects was evaluated by contrast analyses, amalgamating non-significant levels. For all models where species richness was the response variable, we used Poisson errors, corrected for over- or under-dispersion when necessary. Chi-square (χ^2) test was used for Poisson's errors and F test when corrected for over- or

under-dispersion, as recommended by Crawley (2007) and Zuur et al (2009). We evaluated the explained deviance by each significant model, calculating the ratio of explained by total deviance, hence called multiple R^2. If resource addition quantity *per se* affected species richness, we expected that the ANOVA considering only two levels of resource addition ("no addition" and "addition") should be the model with the highest explained deviance.

To test if resource addition promoted the increase of rare species, we adjusted generalized linear mixed models (GLMM) with random intercept and Poisson errors. Random effects were pitfall set, nested within transect. Number of individuals of each cricket species *per* set was the response variable. We performed two-way analysis of variance (ANOVA), with resource addition (2 levels: "no addition" x "addition") and species identity as explanatory factors, together with the interaction term. The use of mixed effects models, adjusting resource addition as random effect, enabled avoidance of pseudo-replication (Crawley, 2007), by evaluating the effects of resource addition on the abundance of all species in a single analyses. If resource addition affected population size differentially among cricket species, favoring some of them (*e.g.* rare species), and reducing other species, the interaction term (species identity : resource addition) should be significant.

To evaluate if resource addition reduced community evenness, we adjusted generalized linear models (GLM - ANOVA), with resource addition (2 levels: "no addition" x "addition") as explanatory factor and community evenness as response variable, using normal errors. To estimate community evenness, we used Simpson's measure of evenness (Magurran, 2004, $\mathrm{E}_{(1/D)} = \frac{1/D}{S}$): where D represents the probability that two individuals randomly selected from a sample will belong to the same species (or some category other than species), and S represents a total number of species captured.

To evaluate the significance of the explanatory variable, we used stepwise backward model simplification, using the *p-value* to exclude non-significant variables. Adjusted models were subjected to residual analyses, to evaluate the adequacy of the model.

Results

We collected 1,115 individuals belonging to four families and 14 species. The richest and most abundant family was Phalangopsidae (eight species and 765 individuals), followed by Trigonidiidae (three species and 333 individuals), Eneopteridae (two species and 3 individuals), and Mogoplistidae, which had only one species and 14 individuals (Table 1).

Using linear regression we detected an increase in cricket species richness with resource addition quantity ($F_{1,34} = 8.48; P = 0.006; R^2 = 20.30\%$), but there was no effect of the quantity of resource added, when the comparison was restricted to samples with resource addition (excluding "no addition" samples; $F_{1,28} = 0.095; P = 0.76$). Using ANOVA, we detected no difference among added resource levels ($F_{4,30} = 1.77; P = 0.16$), but there was an effect of resource addition *per se*: cricket species richness was higher when resource was added, compared to "no addition" ($F_{1,34} = 33.52; P < 0.0001; R^2 = 49.41\%$). Therefore we choose the ANOVA model, with two levels of resource: "no addition" and "addition" (Figure 1).

Table 1 List of taxa sampled by each treatment level of resource addition (zero to five), with their partial and total abundances. The taxa which not present a genus and species valid was collect first time in Brazil are new for science. The manuscript to description genus and species are in preparation.

	Quantity of resource added *per* pitfall-set					
TAXA	**0**	**1**	**2**	**3**	**4**	**5**
Ectecous sp.1	45	88	61	94	145	130
Aracamby sp.1	17	19	12	19	36	27
Phoremia zefai	3	38	22	36	62	54
Zucchiella matiottiae	-	14	12	15	27	14
Amanayara sp.1	-	7	1	6	12	10
Laranda sp.1	-	5	2	13	7	10
Adelosgryllus rubricephalus	-	4	2	2	11	2
Mogoplistidae	-	4	4	1	0	5
Aracamby sp.2	-	3	-	-	-	2
Eidmanacris tridentata	-	1	1	0	0	3
Endecous sp.1	-	1	-	-	-	2
Eneoptera surinamensis	-	1	1	-	-	-
Tafalisca sp.1	-	-	1	-	-	-
Eidmanacris bidentata	-	-	-	1	-	-
Individuals	65	185	119	187	300	259

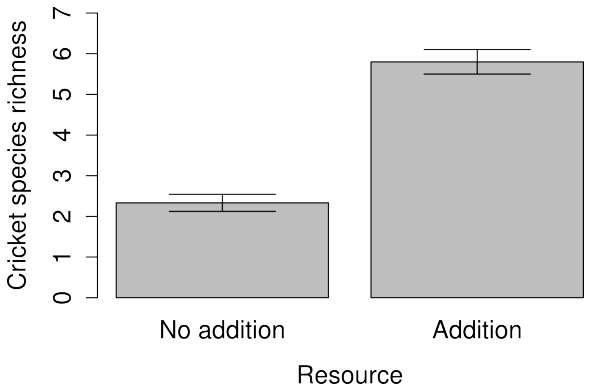

Fig. 1 Number of cricket species against resource levels. Samples where resource was added captured more species than samples where resource was not added. ANOVA - $y = e^{(0.847+0.910*x)}; F_{1,34} = 33.52; P < 0,0001; R^2 = 49.41\%$.

Resource addition affected cricket's abundance differentially among species: using GLMM we detected a significant interaction of species identity with resource addition, evaluated as factor with two levels ($\chi^2 = 49,49; P < 0.001$; Figure 2). The abundance of all cricket species captured in the "no addition" plots was not reduced by resource addition. The less abundant species in the "no addition" plots, *Phoremia zefai*, increased its abundance three times. Eleven cricket species were exclusively captured when resource was added. Therefore, in sites with resource addition, there were higher abundances of rare species.

The third most abundant species in "no addition" samples – *Phoremia zefai* – was the second most abundant species in samples were resource was added, increasing its abundance three times. While most species were favored by resource addition, *Aracamby* sp. 1, which was the second most abundant species in "no addition" samples, was almost not affected, as depicted by the overlapping standard errors (Figure 2).

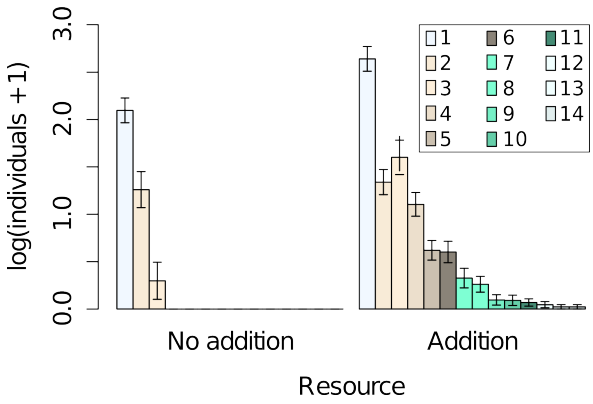

Fig. 2 Resource addition affected cricket abundance: there were a significant interaction among species identity and resource addition. While in "no addition" samples were captured three species and 65 individuals, in the "addition" samples were captured 14 species and 1050 individuals. When resource were added, *Phoremia zefai* increased its abundance three times compared to "no addition" samples. Except *Aracamby* sp. 1, all species were affected by resource addition. In the figure legend, the numbers "1" to "14", represent the species captured in the experiment, as presented in Table 1. GLMM - $\chi^2 = 49,49; P < 0.001$.

Using ANOVA with two levels of resource ("no addition" and "addition") levels, we detected no effect of resource addition on evenness ($F_{1,34} = 2.14; P = 0.15; R^2 = 5.94\%$), but there was an influential outlier, whose deletion altered the result, rendering significant reduction of community evenness when resource was added ($F_{1,33} = 17.91; P = 0.0001; R^2 = 35.18\%$; Figure 3). Therefore we choose the second model, with higher R^2.

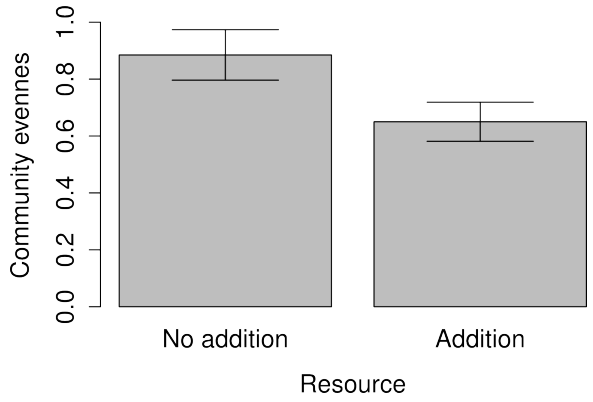

Fig. 3 Community evenness against resource ("no addition" and "addition") levels. When resource was added there was a significant reduction in community evenness. ANOVA - $y = e^{(0.884-0.234*x)}; F_{1,33} = 17.91; P = 0.0001; R^2 = 35.18\%$.

Discussion

Our results show that resource availability is a short-term driver of litter cricket communities. Our results are evidence that 12 of the 14 cricket species present in forest litter are maintained at low densities

by resource scarcity. When resource availability is experimentally increased, species richness increases due to behavioral displacement, changing community structure.

Although litter forest crickets do not use sugarcane as their regular resource, our results showed that sugarcane syrup works as an efficient cricket bait. We consider that this occurred because the crickets perceived sugarcane syrup as a sugar-rich resource, similar to eventually available fallen fruits. Therefore, our results may evidence that sugar-rich resources, such as fruits, may be important food source for litter crickets.

In this study, overall sampling efficiency was *ca.* 30% higher than in a similar study which did not use resource addition, as far as we captured a mean of 6.2 individuals per pitfall trap, compared to 4.9 in Szinwelski et al (2012). The higher sampling efficiency of this study was due to resource addition: the samples with added resources captured a mean of seven cricket individ uals per sample, *i.e.*, more than three times the mean individuals *per* trap in no-addition traps (2.16). We captured only two further species, beyond those captured in a 3.6 times lower sampling effort (50 pitfall-traps, in Szinwelski et al (2012)). This can evidence cricket species present in forest litter are limited by resource scarcity. When resource availability is experimentally increased, species richness increases due to behavioral displacement, changing community structure. We suggest that the cricket species richness estimated in this study has reached the plateau of the species accumulation curve (compare to Figure 7 in Szinwelski et al (2012)). There are still cricket species in this forest that we did not capture. In a huge sampling-effort, done for taxonomy purposes, using hand-sampling together with pitfall-traps, Francisco A. G. de Mello (UNESP) and Pedro G. Dias (USP) (personal communication) collected 25 cricket species, including all 14 captured in our study. Among the 11 additional species, several are rarely captured in pitfall traps, because they live predominantly on tree trunks or canopy.

The increase in species richness in response to resource addition is expected by the idea that productivity drives diversity (Cardinale et al, 2009). We did not evaluate demographic long-term effects, but showed that in the short-term, at least, forest litter crickets appear to be limited by sugar-rich resource availability.

Our resource manipulation was a pulse experiment (Bender et al, 1984; Gotelli and Ellison, 2004), as far as we increased instantaneously the local availability of sugar-rich resource, but did not maintain the experimental change of the environment. Our results show that resource availability is a short-term driver of litter cricket communities. Sugarcane syrup addition promoted a quick response of the litter cricket community, as far as the time during which the crickets were in contact with the changed environment was of just 48 hours. The added sugarcane syrup may have remained in the experimental availability along the whole pitfall sampling period, or it may have decreased it's availability, due to resource consumption. We discarded resource runoff through rainfall, because the was no rain during our experiment. Therefore, the observed effects of resource addition were not due to demographic processes (natality and mortality), but due to behavioral responses. The addition of sugarcane syrup promoted an aggregation of nearby cricket individuals, that otherwise were scattered along a large forest litter area, with lower densities.

We interpret the species richness increase in response to resource addition, as resulting from an increase in the abundance of rare species. The species that where absent from the "no-addition" samples, were probably present nearby, enabling them to respond quickly to the resource addition by behavioral aggregation. Therefore, the aggregation promoted by the experimental resource addition enabled an increase in sampling efficiency, so that a larger portion of the forest cricket species pool was captured.

The species composition in "no addition" samples was a subset, nested within the species composition in the resource "addition" samples. This, added to the fact that when resource was added, the number of captured individuals increased, could correspond to a shift in Preston's veil line (Preston, 1962). When a larger portion of the community was sampled, the probability of capturing rare species increased. A shift in the veil line is, however, not enough to explain our results. Resource addition promoted a change in the community evenness and in the relative abundance of the species.

Although all species presented increased abundance in the "resource addition" samples, *Aracamby* sp. 1 contrasted the overall response, as far as it was almost not affected, as depicted by the overlap of the observed error bars (Figure 2). This suggests that sugar-rich resource availability is not limiting for this species. Either this species does not feed on sugar-rich resources, like fruits, or it is sufficiently efficient in using the available sugar-rich resources, compared to the other cricket species.

The fact that all but one of the 14 litter cricket species were positively affected by sugarcane addition evidences a high food resource overlap among these crickets. When in naturally low availability, sugar-rich resources limit local species densities, maintaining impoverished cricket communities, dominated by the three most abundant species: *Ectecous* sp. 1, *Aracamby* sp. 1 and *Phoremia zefai*. Experimentally increasing resource availability led to a change in the abundance order of the cricket species, with a three-fold increase in abundance of *Phoremia zefai*, which changed from third abundant species in "no addition" samples to second most abundant species in "addition" samples.

Although increase in species richness with resource addition may be due to competitive release promoted by experimentally increased of resource availability, there is an alternative explanation. The cricket species that were attracted by the sugarcane syrup may present feeding preference for this resource, without any competitive interaction with the dominant species in the "no addition" samples.

The reduction of community evenness with resource addition appears contradictory to the observed changes in abundance (see Figure 2). In the "no addition" samples there were only three species, with the remaining 11 species with such low density as to present no captured individuals. This reveals a great difference in the abundance of the three "dominant" species, compared to the 11 rare species. The calculated evenness index, however, does not take absent species into account. More than that, it uses species richness in the denominator, so that increasing the number of sampled species reduces the calculated evenness estimator. Therefore, we suggest that the evenness reduction detected with resource addition reflects rather the increase in species richness than an actual increase in community dominance. For the "no addition" samples, species richness was underestimated, because the rare species present lower densities than those intercepted by the sampling-effort veil line Preston (1962).

Our results highlight that resource is a short-term driver litter crickets on tropical forests. The abundance of many species are maintained low by resource scarcity. When resource were added, the cricket's behavioral displacement promote an increase in species richness and altered species composition. When compared samples with and without resource we found a reduction in a community evenness. However, this can be an mistake due to evenness index. For litter crickets the presence of any additional resource, as sugar-rich for example, can diminished the strength of competition, permitting an increase in abundance and species coexistence.

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4 Capítulo três

4.1 Ethanol fuel improves arthropod capture in pitfall traps and preserves DNA

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Ethanol fuel improves arthropod capture in pitfall traps and preserves DNA

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Abstract

We tested the value of ethanol fuel as a killing solution in terms of sampling efficiency (species richness and accumulated abundance) and DNA preservation of Ensifera ground-dwelling specimens. Sampling efficiency was evaluated comparing abundance and species richness of pitfall sampling using 100% ethanol fuel, with two alternative killing solutions. We evaluated the DNA preservation efficiency of the killing solutions and of alternative storage solutions. Ethanol fuel was the most efficient killing solution, and allowed successful DNA preservation. This solution is cheaper than other preserving liquids, and is easily acquired near field study sites since it is available at every fuel station in Brazil and at an increasing number of fuel stations in the U.S. We recommend the use of ethanol fuel as a killing and storage solution, because it is a cheap and efficient alternative for large-scale arthropod sampling, both logistically and for DNA preservation. For open habitat sampling with high day temperatures, we recommend doubling the solution volume to cope with high evaporation, increasing its efficacy over two days.

Keywords

Killing solutions, molecular tools, taxonomy, large-scale fieldwork, Brazil

Introduction

Several sampling techniques are used to assess biodiversity of different animal species (King and Porter 2005). All present advantages and disadvantages, so the choice is at the discretion of the researcher. Small organisms (e.g. arthropods) are frequently hand-

sampled, which provides information on the organism's habits and behavior, but this method is of little use for ecological comparisons, because of collector interference (Krebs 1999, Southwood and Henderson 2000).

Pitfall traps are a good alternative for collecting ground-dwelling arthropods (Dahl 1896). This kind of trap is inexpensive and easy to handle, allowing both rich and abundant samples. It can be used for taxonomic (although some coloration characters may be lost), ecological, morphological and molecular studies (Gurdebeke and Maelfait 2002, Schoereder et al. 2004, Sperber et al. 2007, Mews et al. 2008, Pereira et al. 2010). One of the main challenges is deciding which killing solution to use in the pitfall traps, which depends on the objectives of each study. As far as sampling involves financial, environmental and researcher's effort costs, the ideal solution should minimize those costs and maximize the utility of the sampled material. The utility of the samples may extrapolate strictly ecological purposes, and should involve other scientific areas, such as morphology and molecular biology. Therefore an ideal should also preserve the specimens' tissues and DNA (Stevens et al. 2011).

Regarding methodological necessities in pitfall sampling, a good killing solution should minimize evaporation, as far as many pitfall trap regimes check traps every 2 weeks or more. A good solution should not be toxic to the researcher nor environmentally harmful. Regarding sampling efficiency, a good solution should kill quickly so as to reduce the escape of specimens. In addition, the trap solution cannot be prohibitively expensive, and must be readily available.

Finding a solution that meets all of these specifications is not easy. Many types of solutions have been used and tested, for example water and detergent, which is inexpensive but accelerates the decomposition of tissues and genetic material (Schmidt et al. 2006). Mixtures of formaldehyde and ethylene glycol (Barber 1931, Sperber et al. 2003b, Schmidt et al. 2006), are efficient in killing and preserving tissue, but are toxic and do not preserve DNA (Aristophanous 2010). Other solutions contain salt brines (Sasakawa 2007) and acetic acid (Gurdebeke and Maelfait 2002), which do not preserve tissues and can alter gonads, genitalia and eggs (Sasakawa 2007). An additional class of solutions contains different concentrations of commercial alcohol (Sperber et al. 2003a, Paquin 2008, Chen et al. 2011), which evaporates faster than the other solutions, but preserves the internal and external organs through tissue dehydration.

It has been shown that at concentrations higher than 95%, commercial alcohol preserves DNA (Nagy 2010), but the use of highly concentrated commercial alcohol as a killing solution may be prohibitively expensive when needed in large quantities, such as in large-scale biodiversity sampling. In Brazil, for example, it is illegal to carry large amounts of commercial alcohol on long journeys, which could hinder its use in extensive field expeditions. Here we propose the use of ethanol fuel as a cheaper and logistically feasible alternative.

In Brazil, ethanol fuel and commercial alcohol have some differences. While the alcoholic concentration (92.6 to 93.8%) and the amount of water (6.2 to 7.4%) varies in ethanol fuel, in commercial alcohol the alcoholic concentration (92.8%) and the amount

of water (7.2%) is fixed. The largest difference is, however, the quantity of gasoline present in ethanol fuel (up to 30 milliliters per liter), that is absent in commercial alcohol (BR0029 2011). In the United States, the highest concentration of ethanol fuel includes 85% ethanol and 15% gasoline (Tatum 2010). Ethanol fuel is available throughout Brazil, at all fuel stations, and at an increasing number of fuel stations in the U.S. (Méjean and Hope 2010, Sorda et al. 2010) and is at least 50% cheaper than commercial alcohol.

In this study, we tested the value of ethanol fuel as a pitfall trap killing solution in terms of sampling efficiency (richness and abundance) and DNA preservation of Ensifera ground-dwelling specimens, comparing 100% ethanol fuel with two alternative killing solutions.

Material and methods

Sampling efficiency

Field sampling site

To evaluate sampling efficiency, we conducted field sampling in a primary Atlantic Forest reservoir, the Iguaçu National Park, in Foz do Iguaçu municipality (25°32'S, 54°35'W, 195m above sea level), Paraná State, in January 2010. The vegetation is mostly tropical semideciduous forest and Araucaria forest, within the Atlantic Forest biome (Rizzini 1997, Dias et al. 1998). The climate is mesothermal subtropical superhumid, with average annual temperatures between 18 and 20 °C and an average rainfall of 1600mm (Peel et al. 2007).

Sampling design

We compared the efficiency of 100% ethanol fuel pitfall killing solution (Solution 1) for ground-dwelling Orthoptera, against the conventional killing solution, comprised of 80% commercial alcohol (80°GL) + 10% glycerin (P.A) + 10% formaldehyde (P.A) (Sperber et al. 2003b) (Solution 2), and a solution of 90% commercial alcohol (80°GL) + 10% glycerin (P.A) (Solution 3). GL is the amount, in milliliters, of absolute alcohol contained in 100 milliliters of hydro-alcoholic solution. P.A., or 'Pro Analysis' means that the sample is of a very high purity, sufficient to be used in chemical analyses. Formaldehyde is recommended for better preservation; glycerin is used to prevent stiffening of the sampled specimens.

For this comparison, we designed the following field experiment. We established a transect of 5km, starting at a distance of 100m from the forest's edge. At the beginning of the transect a set of five pitfall traps, containing one of the three killing solutions chosen randomly, were placed perpendicularly to the transect, 2m apart from one another. After the next 30m on the transect, we placed the second set with a different, randomly

chosen, killing solution. After another 30m along the transect, we placed the third set, with the third killing solution. After an additional forty meters we began the procedure again, and repeated it a total of 50 sampling stations. In summary each sampling station contained five pitfall traps with each of the three killing solutions, for a total sampling effort of 750 pitfall traps. Traps consisted of polyethylene vials, 20cm in diameter and 22cm deep, filled with 500ml of killing solution. After 48 hours, specimens were removed from the the traps, identified and stored in ethanol fuel, after gathering the data.

Data analysis

To evaluate sampling efficiency of ethanol fuel as a pitfall killing solution, we compared cricket species richness and accumulated abundance (= total number of individuals per pitfall set) among the three solutions. Each pitfall set was considered one sampling unit, rendering 150 replicates. We performed one-way analysis of variance (ANOVA), adjusting generalized linear models (GLMs) with Poisson error distribution, correcting for over- or under-dispersion using quasi-Poisson when necessary. We considered cricket species richness and accumulated abundance in each set of five pitfall traps as response variables (n = 150), and the type of killing solution as the explanatory factor. We used contrast analyses to evaluate effect differences among the kinds of solution, simplifying the complete models by amalgamating non-significantly different factor levels (Crawley 2007). We used Chi-square (χ^2) test for Poisson error distributions, and the *F* test in cases where there was a correction for over- or under-dispersion, as recommended by Zuur et al. (2009). We checked residuals for homoscedasticity. All analyses were undertaken within the R 2.15 environment (R Development Core Team 2012).

DNA preservation

Killing and storage

To test the DNA preservation properties of each pitfall killing solution, we placed each of 18 living cricket specimens of *Gryllus* sp. (not identified) into one of the three pitfall killing solutions, totaling six specimens per solution. As a control, we separately placed another six crickets into undiluted commercial alcohol (92.8°GL), which is considered a good preservative of DNA (Nagy 2010). Twenty-four hours later, we took one leg of each individual and extracted its DNA. Twenty-four hours later (*i.e.* 48 hours after immersion into the killing solution), we removed a second leg off the crickets to evaluate DNA preservation, analogous to in the field procedure collecting time of 48 hours, as recommended by Sperber et al. (2003a) for ground-dwelling Orthoptera sampling.

To evaluate the efficiency of ethanol fuel as a storage solution, we stored each cricket specimen, after 48 hours in the killing solution, in one of two storage solutions: undiluted commercial alcohol (92.8°GL) or undiluted ethanol fuel. To test

the effect of time and type of storage solution on the DNA preservation efficiency, we removed a third leg off each cricket after 15 days, and a fourth leg after 30 days in the storage solution.

We evaluated efficiency of DNA preservation for the 24 crickets used in the above procedure. Each set of six individuals was submitted to one of four different killing solutions, and each individual provided two samples (= legs) for DNA extraction before storage (24 and 48 hours in the killing solution). Individuals from each killing solution were transferred to either commercial alcohol or ethanol fuel for storage, providing three replicates (individuals) per storage solution, and two further samples (= legs) per individual, 15 and 30 days in the storage solution. All specimens were maintained at room temperature for 30 days.

DNA extraction

Total DNA was isolated from each individual using the protocol described in Waldschmidt et al. (1997) but without the deproteinization step with phenol:chloroform (1:1). Preliminary analysis of fresh specimens killed by freezing showed that tissue extractions from the thorax or legs were equally effective. Therefore, we chose to use only the legs, allowing maximum preservation of anatomical parts for further studies, and repeated sampling of the same individuals with minimum tissue damage.

DNA extractions were verified via agarose gel (0.8%) electrophorese, prepared and run in 1X TBE Buffer, stained with ethidium bromide and viewed under UV light. The quality of the extractions was checked by comparison with the extract made from fresh material (specimens that were killed by freezing, with immediate DNA extraction). Extractions from fresh material presented two bands, the first clearly marked and bright, corresponding to genomic DNA and the second smaller, more opaque, corresponding to RNA. We considered DNA as properly preserved when we detected a well-defined single band of DNA without apparent trawlers.

Results

Sampling efficiency

We collected 3,528 individuals of 14 species from four different families of Orthoptera, following the classification of Desutter-Grandcolas (1987, 1988): Phalangopsidae (2,090 individuals of eight species), Trigonidiidae (835 individuals of two species), Gryllidae (394 individuals of two species) and Eneopteridae (209 individuals of two species). Species richness ($F_{2,147}$ = 177.09; $p < 0.001$) and abundance ($F_{2,147}$ = 104.64; $p < 0.001$) were significantly higher in pitfalls with ethanol fuel killing solution (Figure 1 A, B) than in those containing the other two solutions. Sampling efficiency was not different between killing solution 2 and 3 (richness: $F_{2,147}$ =0.34; $p = 0.55$; abundance: $F_{2,147}$ = 2.87; $p = 0.09$).

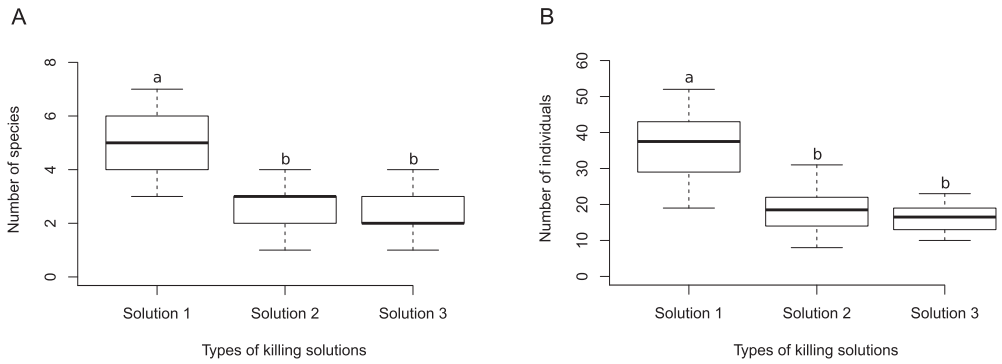

Figure 1. Boxplot showing sampling efficiency of different kinds of pitfall traps' killing solution. Traps with **Solution 1** (100% ethanol fuel) captured more species and individuals than **Solution 2** (80% commercial alcohol (80°GL) + 10% glycerin (P.A) + 10% formaldehyde (P.A)) and **Solution 3** (90% commercial alcohol (80°GL) + 10% glycerin (P.A)). **A** Total number of species per pitfalls' set. **B** Total number of individuals per pitfalls' set. Different lower case letters correspond to significant differences between killing solution levels, evaluated through contrast analyses.

DNA Preservation

Table 1 indicates that both solution 1 and solution 3 were efficient in preserving DNA and are appropriate for use as killing solutions in pitfall traps that must remain in the field for up to 48 hours, with no visible damage to DNA. In addition, these samples can be stored at room temperature for up to 30 days in either commercial alcohol or ethanol fuel. On the other hand, our results suggest that just 24 hours in solution 2 (commercial alcohol + glycerin + formaldehyde) are enough to destroy the DNA of the samples (Figure 2).

Table 1. Success (yes) or failure (no) of DNA extractions after different periods (Time in the solution) in Killing solution (Pitfall: 24h and 48h) and in storage solution (C.A. and E.F.: 15 and 30 days). C.A. = undiluted commercial alcohol (92.8°GL); E.F. = undiluted ethanol fuel; Solution 1 = E.F.; Solution 2 = 80% commercial alcohol (80°GL) + 10% glycerin (P.A.) + 10% formaldehyde (P.A.); Solution 3 = 90% commercial alcohol (80°GL) + 10% glycerin (P.A.). All material was maintained at room temperature. Asterisks mark the treatments shown in Figure 2.

Killing solutions	**Time in the solution**					
	Pitfall		C.A.		E.F.	
	24h	48h	15days	30days	15days	30days
C.A.	yes	yes	yes	yes*	yes	yes*
Solution 1	yes	yes	yes	yes*	yes	yes*
Solution 2	no*	-	-	-	-	-
Solution 3	yes	yes	yes	yes*	yes	yes*

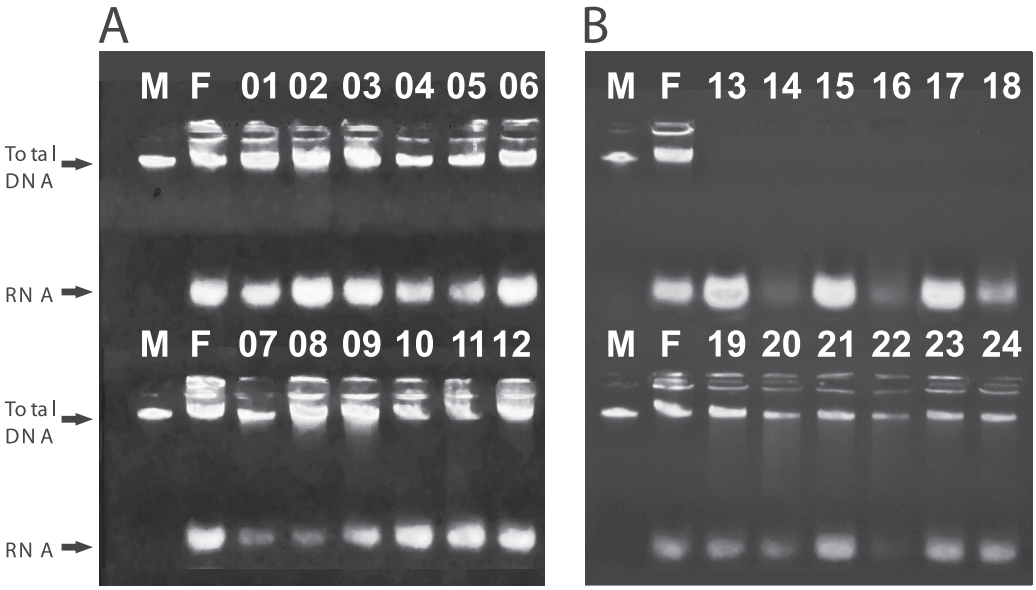

Figure 2. Electrophoresis of all 24 analyzed individuals. M represents the lambda DNA marker (100 ng/ul) and F represents the control extraction made using fresh tissue. A) Lanes 01 – 06, individuals killed in C.A. (undiluted commercial alcohol), maintained in the killing solution for 48 hours and then transferred to closed vials containing C.A. (01 – 03) and E.F. (03 – 06) and maintained in these storage solutions for 30 days. Lanes 07 – 12, individuals killed in Solution 1 (= E.F.), maintained in the killing solution for 48 hours and transferred to C.A. (07 – 09) and E.F. (10 – 12) and maintained in these storage solutions for 30 days. B) Lanes 13 – 18, individuals killed in the Solution 2 and maintained in this solution for 24 hours. Lanes 19 – 24, individuals killed in Solution 3, maintained in this solution for 48 hours, than transferred to C.A. (19 – 21) and E.F. (22 – 24) and maintained in these solutions for 30 days. All DNA extractions where successful, but those of crickets killed in solution 2 (lanes 13 – 18).

Discussion

In this study, we investigated the efficiency of ethanol fuel as a pitfall killing solution in terms sampling efficiency, as measured by species richness and accumulated abundance, and in terms of DNA preservation. Our results indicate increased sampling and preservation efficiency of ethanol fuel, compared to the commonly used alternatives. Below we discuss the advantages and disadvantages of using ethanol fuel as a pitfall killing and storage solution, with particular emphasis on large-scale field expeditions.

Financial costs

Of the solutions tested in our study, ethanol fuel is the least expensive option: 1 liter of ethanol fuel (US$ 1.25 on average) costs less than half the price of 1 liter of commercial alcohol (US$ 3.15), which does not include the other components, such as glycerin and formaldehyde, which cost around US$ 15.00 a liter (prices for Brazil).

Field logistics

The transportation of flammable or toxic liquids is dangerous and illegal under Brazilian and international law. This danger increases with the distance, and consequently time spent in transportation. Ethanol fuel presents a partial solution to this limitation: as it can be bought near the field study sites, at any fuel station in Brazil, the distance of transportation is diminished, decreasing the danger. Large field expeditions can use these facilities to reduce the distances of ethanol transportation, thus reducing the risks of accidents, and simplifying expedition logistics. Even so, for transportations and storage of collected material, we recommend using firm, pressure-resistant bottles, with sealed caps, fully filled with ethanol, so as to minimize oxygen within the bottle, reducing explosion risks. We used PET tubes, which have low costs and may be bought in large quantities.

Commercial alcohol has to be purchased in large shops when bought in large quantities, and is hardly available in the small towns that border most of the large conservation areas. Therefore it would require long-distance transportation and represent huge environmental and personal risks. The additional components of the tested killing solutions (glycerin and formaldehyde), are only available in specialized establishments, restricted to a few large cities in Brazil (Brazilian Federal Law n°10.357/2001).

Sampling efficiency

We showed that ethanol fuel presented higher sampling efficiency, both for species richness and accumulated abundance of ground-dwelling Orthoptera species, therefore maximizing the gains of the sampling effort. We hypothesize that this higher sampling efficiency is related to the lower density and surface tension of the solution 1 (density = 0.81 g/cm^3; surface tension = 21.55 mN/m^{-1}) than solution 2 (density = 0.92 g/cm^3; surface tension = 48.56 mN/m^{-1}) and solution 3 (density = 0.97 g/cm^3; surface tension = 55.34 mN/m^{-1}) (Adamson and Gast 1997), which could cause the crickets to sink and die faster in ethanol fuel, reducing their chances of escape from the trap.

One piece of evidence in favor of our hypothesis is that all winged cricket species captured in this study died exclusively within pitfalls that used ethanol fuel as the killing solution (94 individuals of *Eneoptera* sp. and 183 individuals of *Gryllus* sp.). These genera contain species of large body size, which are powerful jumpers as nymphs and powerful fliers as adults, and are rarely captured in conventional pitfall traps killing solution (N. Szinwelski, personal observation). Indeed, C.F. Sperber, in other field collections, has observed adults of *Eneoptera* sp. flying out of pitfalls with water + detergent killing solution. The alternative pitfall design used to prevent escape from traps, using an inverted funnel at the trap's top (Melbourne et al. 1997), may reduce sampling efficiency, especially for good jumpers and fliers.

DNA preservation efficiency

To obtain DNA samples, it is recommended that the sampled organisms be removed from the pitfall killing solution as soon as possible and placed in vials containing highly concentrated alcohol, preferably at low temperatures (Nagy 2010). Based on the results presented here, we suggest that sampled organisms may be safely stored in undiluted ethanol fuel at room temperature, without major damage to DNA quality, for up to 30 days.

Indeed, we were able to obtain sequences of mitochondrial DNA (COI) and nuclear (18S rRNA) of Orthoptera specimens kept for two weeks in ethanol fuel killing solution, before being sorted and stored in undiluted commercial ethanol (92.8°GL), where they remained at 38°C – 45°C room temperature for another 45 days (in Manaus – AM) and 70 days at similar temperature (in Cuiabá – MT).

Counterarguments

One of the main arguments against the use of ethanol fuel as a pitfall trap killing solution is that it evaporates faster than other solutions, making its use limited to high temperature areas. We were, however, able to use ethanol fuel pitfall traps successfully in Amazon forest sampling (38°C – 45°C), where the traps were kept for 48h in the field without significant volume reduction of the killing solution.

Solution evaporation is a limiting factor in open habitat with high temperatures as Brazilian "Campo Cerrado", for example. In such field conditions, we recommend increasing the killing solution volume by 100%, from 500ml to 1000ml, to maintain sufficient killing solution volume in the traps after 48h in the field.

Another problem with ethanol fuel is the fact that it can be denatured. In Brazil, that means that every liter of ethanol fuel can contain up to 30ml of gasoline. In the United States every liter of ethanol E85 contain 150ml of gasoline. This may represent an environmental problem if the pitfall is damaged and the solution is spread in the environment. Moreover, gasoline might hinder DNA preservation. For Brazilian ethanol fuel we showed that this did not occur. Even specimens collected in ethanol fuel, were successfully preserved and we were able to extract DNA and run PCR reactions obtaining sequences of mitochondrial COI and nuclear rRNA18S .

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5 Capítulo quatro

5.1 Ethanol fuel improves pitfall traps through rapid sinking and death of captured organisms

This article was submitted to **Environmental Entomology**, and is under review.

Ethanol fuel improves pitfall traps through rapid sinking and death of captured organisms

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Abstract

The choice of killing solutions for pitfall traps can influence sampling and is highly dependent on the objectives of each study. Nevertheless, it is increasingly common to use the same organisms in different kinds of studies. The killing solution should be able to sample local active organisms, as well as maintain the integrity of their organs, tissues and macromolecules. In a previous work, we showed that using ethanol fuel as a killing solution maintains the integrity of the specimens and enhances the richness and abundance of Orthoptera species sampled. In the present study, we aimed to assess if ethanol fuel sampling is sufficient, *i.e.* if it collects the entire species spectrum sampled by other killing solutions. We also set up a field experiment to test whether the ethanol fuel attracts Orthoptera species. We investigated in the laboratory whether individuals of *Gryllus* sp. sink or die faster in ethanol fuel than in other solutions. Our results allowed us to refute the hypotheses of attraction or repellency caused by ethanol fuel and showed the highest sampling efficiency of this compound is directly linked to the specimens sinking and dying faster than in other killing solutions. Thus, in addition to taxonomic, anatomical and molecular studies, ethanol fuel can also be used in ecological studies to exclusively sample local active organisms, at least for species of Orthoptera.

Introduction

Pitfall traps are widely used in ecological studies for collecting ground-dwelling arthropods [4–9], but the data obtained with these traps should be interpreted with caution. Several trap characteristics affect sampling results, such as diameter, layout and construction material, color, bait and preservatives [1, 2], which can influence the data and lead to erroneous conclusions [3]. Therefore, each of these factors must be taken into account when selecting the most appropriate type of pitfall trap to achieve adequate and unbiased sampling, according to the target organism group and the goal of the study.

Prior to 2003, pitfall traps were filled with solutions of water, detergent and salt, at different concentrations [4]. These killing solutions were inadequate for unbiased sampling, because they underestimated the abundance of mobile, large and fully winged individuals, such as some crickets, which were observed

escaping from pitfall traps (C. F. Sperber, pers. obs). Additionally, these killing solutions were inadequate for preserving organisms, as those captured degraded rapidly, making species identification difficult or even impossible. This also the case with molecular studies as the killing solutions damaged and degraded DNA [10].

In 2003, a killing solution for cricket sampling composed of 80% commercial alcohol (80°GL) + 10% glycerin (P.A.) + 10% formaldehyde (P.A.) was proposed [4]. This solution is more efficient as a killing and preservative solution because it retains more adults because of its knockdown effect. This killing solution, however, presents some shortcomings for taxonomic studies, since it can distort genitalia sclerites, and destroys the DNA of sampled individuals after only 24 hours [8].

In an attempt to overcome some of these drawbacks, we recently proposed replacing the previous solutions with 100% ethanol fuel [8]. Our results were satisfactory, since we obtained significantly more species richness and abundance with ethanol fuel than traditional killing solutions. Our results were satisfactory, since we obtained significantly more species richness and abundance with ethanol fuel than traditional killing solutions. Moreover, we show that ethanol fuel, despite containing gasoline, adequately preserves the DNA and morphology of captured individuals. For ecological studies, however, the use of ethanol fuel as a killing solution could be problematic if it repels or attracts individuals by its odor.

In this work, we first revisited our previous results [8] to evaluate if (i) sampling with ethanol fuel is sufficient, *i.e.*, whether it collects the whole species spectrum sampled by other solutions, or if some species are repelled. Aside from this, our main question is: why do ethanol fuel samples present higher species richness and accumulated abundance than other killing solutions? We tested the hypotheses that (ii) ethanol fuel is attractive and that (iii) ethanol fuel reduces escape, through faster sinking or death than other killing solutions.

Materials and Methods

Is ethanol fuel sampling sufficient?

Revisited data

To evaluate if ethanol fuel sampling is sufficient, *i.e.*, if it collects the whole species spectrum sampled by other killing solutions, we compared the species spectrum obtained using three different killing solutions tested in our previous article [8]: 100% Ethanol fuel (solution 1); 80% Commercial alcohol (80°GL) + 10% Glycerin (P.A) + 10% Formaldehyde (P.A) (solution 2) [4], and 90% Commercial alcohol (80°GL) + 10% Glycerin (P.A) (solution 3). If ethanol fuel sampling is sufficient, we expected that the species spectrum sampled with this solution would contain all species sampled with the other solutions. Full details about sampling procedures are available in our previous paper [8].

Statistical analysis

To evaluate if ethanol fuel sampling is sufficient, we performed NESTEDNESS analysis. The NESTEDNESS null hypothesis is that there species assemblages present nested patterns, in which species

compositions of small assemblages comprise a nested subset of larger assemblages [11]. If ethanol fuel sampling is sufficient, we expected a perfectly nested pattern, with ethanol fuel capturing the whole species spectrum, and the other killing solutions capturing smaller species assemblages nested within the killing solution assemblage.

We measured the degree of nestedness [11,12], with the 'vegan' package [13] within the R environment [14]. We calculated the NODF (Nestedness metric based on Overlap and Decreasing Fill) statistics [15], running 10,000 simulations, using the 'r1' method, which uses both row and column constraints [11]. The NODF statistics vary from 0 to 100, with 100 representing maximum nestedness [16]. The software holds as null hypothesis the nestedness pattern. Hence, when we cannot reject the null hypothesis, a nested pattern is present on the data.

Is ethanol fuel attractive?

Field manipulative experiment

To evaluate the hypotheses that ethanol fuel is attractive, we run a manipulative experiment in the field, in a remnant of semi-deciduous Atlantic forest, the Mata do Paraíso Research Center (MPRC) ($20°41'20''S - 20°49'35''W$), Viçosa municipality, Minas Gerais State, Brazil [17] in February 2012.

In this experiment, we tested the attractiveness of four different attractive solutions: water, commercial alcohol (92.8 °GL), 100% ethanol fuel and sugar cane juice. We used polyethylene vials of 20 cm in diameter and 22 cm deep as pitfall traps, filled with 500 ml of a killing solution comprised of water + 2.5% neutral detergent. In each pitfall trap we attached two PET tubes (2 cm in diameter, 15 ml each), glued to the inside of the trap with gluing tape, 180° from each other, containing one of the four attractive solutions.

We established a transect of 1700 m, starting at a distance of 200 m from the forest edge. At the beginning of the transect, a set of four pitfall traps, each containing one of the four attractive solutions chosen at random, was placed perpendicular to the transect, 30 m apart from one another. After the next 50 m on the transect, we placed another set of pitfall traps, repeating this procedure a total of 30 times, with each site containing one pitfall trap for each of the four attractive solutions, for a total sampling effort of 120 pitfall traps. The pitfall traps were left in the field for 48 h. Afterward, the traps were removed and the specimens identified and stored in ethanol fuel. Voucher specimens were deposited in the Laboratory of Orthoptera, affiliated with the *Museu Regional de Entomologia da Universidade Federal de Viçosa* (UFVB).

Of the four attractive solutions, we consider water the negative control, *i.e.*, it should have little or no attractive effect. We used commercial ethanol to distinguish whether the attraction is caused by ethanol *per se* or by traces of gasoline contained in ethanol fuel (in Brazil, ethanol fuel contains up to 30 ml per liter of gasoline, BR0029 2001). Finally, we used sugarcane juice as a positive control, since it is widely used as an attractive solution, to increase Orthoptera sampling efficiency [18].

If ethanol fuel is attractive, we expected higher numbers of individuals and species in pitfalls with this solution in the attached vials than in the negative control. The null hypothesis was that pitfalls with ethanol fuel in the attached vials would capture the same number of individuals and species as

the negative control. The positive control was used to test if our experimental device was sufficient to promote attraction: in this case, traps with sugarcane juice should capture more individuals and species than the negative control.

Statistical analysis

To test the hypotheses that ethanol is attractive, we compared the number of species and individuals per pitfall trap among the four attractive solutions. Each pitfall trap was considered one sampling unit, so the 120 pitfall traps rendered 30 replicates *per* treatment level. We performed one-way analysis of variance (ANOVA), adjusting generalized linear models (GLMs) with Poisson error distribution and corrected for over- or under-dispersion using quasi-Poisson when necessary. We considered cricket species richness and abundance in each pitfall trap as the response variable, using separate statistical models. The type of attractive solution was adjusted as an explanatory factor with four levels. We used contrast analyses to evaluate the differences among types of attractive solutions, simplifying the complete model by amalgamating non-significantly different factor levels [19]. We used the Chi-square (χ^2) test for Poisson error distributions, and the F test when corrected for over- or under-dispersion [20]. All models were subjected to residual analyses, and all analyses were done using the R 2.12.1 environment [14].

Does ethanol fuel reduce escape?

Laboratory experiment

To test the hypotheses that ethanol fuel reduces escape by quicker sinking or killing of the captured individuals, we compared the three killing solutions used in our previous work [8]. We carried out the following manipulative experiment in the laboratory, using *Gryllus* sp. reared in the Laboratório de Orthoptera - UFV. We prepared three pitfall trap vials, each with 500 ml of one of the following killing solutions: 100 % Ethanol fuel (solution 1); 80 % Commercial alcohol (80 °GL) + 10 % Glycerin (P.A) + 10 % Formaldehyde (P.A) (solution 2), and 90 % Commercial alcohol (80 °GL) + 10 % Glycerin (P.A) (solution 3).

Therefore, we placed the vials with the killing solutions on a white surface table to facilitate visualization. To simulate individuals natural falling into the pitfall trap, we dropped a cricket from the edge of the vial into it, marking the time that crickets took to sink (= touch the bottom of the pitfall) and die (= complete immobilization). After death, we removed the cricket from the vial, labeled it and stored it. We repeated this procedure 30 times for each killing solution, using 90 adult individuals.

Statistical analysis

To test the hypotheses that ethanol fuel reduces escape by sinking or killing captured individuals more quickly, we compared the time of sinking and dying among killing solutions 1, 2 and 3. Each cricket was considered one sampling unit, rendering 30 replicates per treatment level. We performed one-way analysis of variance (ANOVA), adjusting generalized linear models (GLMs) with normal error distribution and the F test. We considered time of cricket sinking and dying as response variables in separate statistical

models, and the type of killing solution as the explanatory factor, with three levels. We used contrast analyses to evaluate the differences among the types of attractive solution, simplifying the complete model by amalgamating non-significantly different factor levels [19]. All models were subjected to residual analyses, and all analyses were done using the R 2.12.1 environment [14].

Results

Is ethanol fuel sampling sufficient?

NESTEDNESS analysis revealed a nested pattern ($NODF = 34.96; p = 0.94$), with all species collected in killing solutions 2 and 3 comprising a a subset of the richest group of species collected by ethanol fuel killing solution (Table 1).

Table 1. Taxa sampled in a field experiment designed to compare the captured species spectrum of three different killing solutions [8]. Solution 1 (100 % ethanol fuel) captured the whole species spectrum, while the killing solution 2 [80 % Commercial alcohol (80°GL) + 10 % Glycerin (P.A) + 10 % Formaldehyde (P.A)] and 3 [90 % Commercial alcohol (80°GL) + 10 % Glycerin (P.A)] captured smaller species assemblages. NESTEDNESS analysis showed a perfectly nested pattern (NODF = 34.96; p = 0.94).

	Killing solutions		
Taxa	**Solution 1**	**Solution 2**	**Solution 3**
Adelosgryllus rubricephalus	X	X	X
Aracamby sp.1	X	X	X
Aracamby sp.2	X	X	X
Ectecous sp.1	X	X	X
Eidmanacris tridentata	X	X	
Endecous sp.1	X		
Eneoptera surinamensis	X		
Gryllus assimilis	X		
Laranda sp.1	X	X	X
Lerneca sp.1	X	X	X
Miogryllus sp.1	X	X	X
Phoremia rolfsi	X	X	X
Tafalisca sp.1	X	X	
Zucchiella matiottiae	X	X	X

Field manipulative experiment: Is ethanol fuel attractive?

We collected 393 individuals of eight species from three families of Orthoptera: Gryllidae (one species and 7 individuals), Phalangopsidae (four species and 122 individuals) and Trigonidiidae (three species and 264 individuals) (Table 2).

Species richness ($F_{1,119} = 13.81; p < 0.001$; Figure 1 A) and abundance ($F_{1,119} = 5.89; p < 0.001$; Figure 1 B) *per* trap were higher in traps with sugarcane as attractive solution than in those containing the other attractive solutions. There was no difference in attraction *per*trap among commercial alcohol,

Table 2. Number of individuals of each *taxa* sampled in a field experiment to test attractiveness of four solutions: sugarcane (positive control), commercial alcohol, ethanol fuel and water (negative control). As expected, sugarcane was attractive: despite all having water for the killing solution, pitfall traps with sugarcane as attractive solution captured more individuals and species, than pitfall traps with commercial alcohol, ethanol fuel and water, as attractive solutions.

	Attractive solution			
TAXA	**Sugar cane**	**Commercial alcohol**	**Ethanol fuel**	**Water**
Ectecous sp.1	32	24	38	11
Eidmanacris sp.1	4	3	1	4
Endecous sp.1	2	-	-	-
Gryllus sp.1	4	3	-	-
Mellopsis doucasae	3	-	-	-
Phoremia Rolfsi	28	18	7	19
Phoremia zefai	39	35	36	30
Zucchiela matiotiae	21	16	6	9
Total	133	99	88	73

ethanol fuel and water attractive solutions (richness: $F_{1,118} = 0.86; p = 0.35$; abundance: $F_{1,118} = 3.06; p = 0.08$).

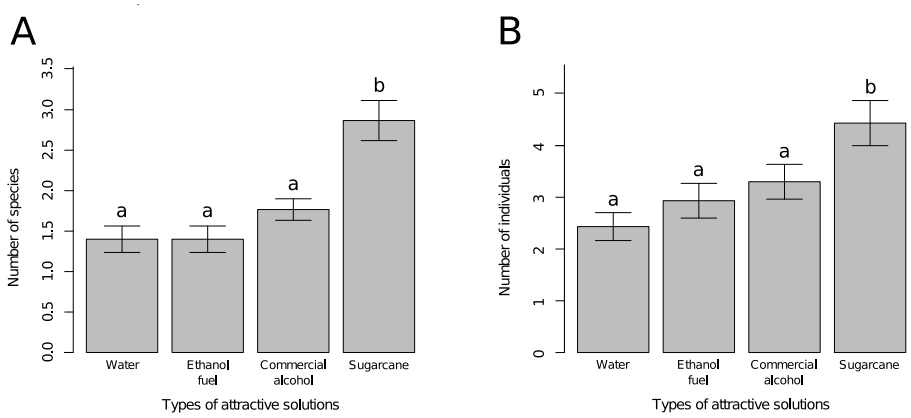

Figure 1. Barplot showing the attractiveness efficiency of four different types of attractive solutions *per* pitfall trap. Traps with the sugarcane as attractive solution captured more species ($F_{1,119} = 13.81; p < 0.001$) and individuals ($F_{1,119} = 5.89; p < 0.001$) than water, ethanol fuel, and commercial alcohol. **A.** Number of species *per* pitfall trap. **B.** Number of individuals *per* pitfall trap. Different lower case letters correspond to significant differences between killing solution levels, evaluated through contrast analyses.

Laboratory experiment: Does ethanol fuel reduces escape?

The sinking time was lower in ethanol fuel ($F_{1,88} = 27.15; p < 0.0001$), but similar between solutions 2 and 3 ($F_{2,87} = 2.22; p = 0.13$; Figure 2 A). Time of death was lower in ethanol fuel, followed by solutions 2 and 3 ($F_{2,87} = 81.30; p < 0.0001$; Figure 2 B).

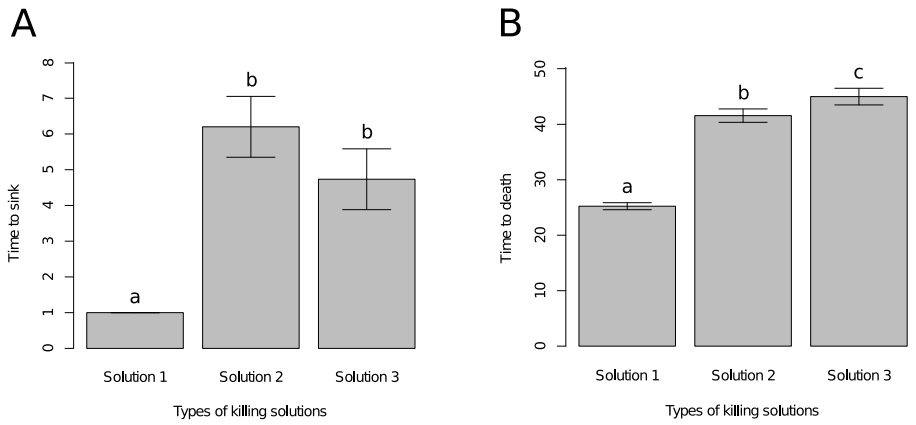

Figure 2. Barplot showing the time to sink and death of three different types of killing solutions. **A.** In solution 1 (100 % ethanol fuel) crickets sink faster than solution 2 [80 % commercial alcohol (80°GL) + 10 % glycerin (P.A) + 10 % formaldehyde (P.A)] and 3 [90 % commercial alcohol (80°GL) + 10 % glycerin (P.A)] ($F_{1,88} = 27.15; p < 0.0001$). **B.** In solution 1 crickets died 40 % faster, followed by solution 2 and 3 ($F_{2,87} = 81.30; p < 0.0001$). Different lower case letters correspond to significant differences between killing solution levels, evaluated through contrast analyses.

Discussion

In Szinwelski et al. [8], we show that ethanol fuel, besides preserving DNA molecules, improves both richness and abundance of Orthoptera capture. However, it remained unknown whether ethanol fuel repels or attracts species of Orthoptera and explain why this solution samples a higher species richness and abundance compared to killing solutions traditionally used in ecological studies.

Our results allowed us to refute the possible repellency of ethanol fuel killing solution, since it sampled the full species spectrum detected in other killing solutions and included under-sampled *taxa*. In other words, in addition to collecting the same groups sampled by other killing solutions, ethanol fuel also captures species that are rarely or never sampled otherwise (Table 1). Based on our experiment, we also refute the potential attractiveness of ethanol fuel, since it was as attractive as water alone. Therefore, ethanol fuel as a killing solution is ideal for correlational studies that investigate local environmental drivers of cricket biodiversity.

Rather than attract, ethanol fuel makes crickets sink very quickly (in less than one second), decreasing

the chances of escape. With this, we hope that all specimens that fall into the trap and have contact with the ethanol fuel are effectively collected, contrary to what occurs in other solutions where animals that can fly or jump (as do many species of Orthoptera) have a chance to escape. The quick sinking is due to lower surface tension of ethanol fuel, compared to the other killing solutions [8].

To illustrate the advantages of rapid sinking, cricket genera such as *Eneoptera*, abundant in the Pantanal litter (N. Szinwelski, pers.obs.) or *Gryllus* and *Tafalisca*, abundant in early regeneration Atlantic forest litter [9], are good jumpers and flyers. Regardless of their abundance, they were rarely captured in pitfall traps with non-ethanol fuel killing solution, except for immature individuals lacking developed wings. Immatures are difficult to identify because they do not present developed genitalia. Besides, when properly identified, these *taxa* were represented mostly by singletons, *i.e.*, only one captured individual. Though singletons may be unavoidable, especially in hyper-diverse habitats [21], they are interpreted as the result of species rareness, which is not the case if the taxon is simply under-sampled. Also, singletons are a difficult statistical problem [21–29], which affects estimates of species richness and may lead to misleading conclusions.

In addition to causing organisms to sink quickly, ethanol fuel kills 40 evaluated here. This makes the use of ethanol fuel as a killing solution ethically mandatory, as it minimizes the organisms eventual suffering. In addition, individuals that sink quickly but struggle a lot and take a long time to die may damage their own body parts and those of other individuals. This is particularly common for autotomy behavior organisms like Orthoptera that drop their hind legs to avoid predation. Small vertebrates (reptiles and small mammals) that fall into the trap might also die quickly, which is advantageous since these animals have the potential to completely damage the samples in a trap. Damaged individuals may be more difficult to identify and are of restricted use in taxonomic studies.

The better the organisms are preserved, the more useful they will be for morphological, molecular [8] and stable isotope studies [30]. Multiple studies on the same sample are a current trend in science, for two main reasons: (i) with the accelerating degradation of biodiversity, the availability of natural habitats and their organisms is decreasing exponentially, making the sharing of biological data mandatory to minimize the ecological impact of sampling effort; (ii) multiple studies on the same samples allows data crossing and result comparisons, favoring the scientific contribution of such interconnected studies.

Regardless of the positive results obtained in Orthoptera studies using ethanol fuel, the use of this killing solution must be studied for each insect order. For example, in Scarabaeinae, Scolytidae and Staphilinidae, this solution should be avoided due to the attractive effect that ethanol has on these groups [31–33], when the goal is to capture locally active organisms. When using baits, however, the attractive effect of ethanol fuel may enhance sampling efficiency, in addition to maximizing morphological and molecular preservation [34].

We therefore conclude that ethanol fuel is neither attractive nor repellent for cricket species, and the higher species richness and abundance in pitfall traps with this killing solution is due to quicker sinking and death of the individuals that fall into the trap. Ethanol fuel captures a larger species spectrum than other killing solutions, including species that are generally under-sampled. Therefore, we strongly recommend the use of ethanol fuel as a killing solution for sampling and storing collected individuals for both scientific and ethical reasons.

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Author Contributions

Conceived and designed the experiments: NS KSCY LRS. Performed the experiments: NS KSCY LRS. Analyzed the data: NS RRS LRS CFS. Wrote the paper: NS KSCY RRS CFS.

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6 Conclusões Gerais

A exploração desordenada de áreas áreas naturais, especialmente áreas florestais, tem contribuído para o aumento da extinção de diversos animais e plantas, muitos, sem que a ciência tenha conhecimento. Historicamente, a floresta Atlântica tem sofrido ampla ação antrópica, restando, atualmente, menos de 5% da cobertura florestal original. A floresta Atlântica apresenta alta biodiversidade, além de muitas espécies endêmicas, e é considerada uma das regiões que apresentam maior biodiversidade (*hotspots*) e, portanto, maior necessidade de proteção e conservação. A fragmentação dos ambientes naturais impede que várias espécies tenham uma área mínima para alimentação e reprodução. Muitas vezes, a falta de conhecimento sobre o que, aonde e como preserva pode levar muitas iniciativas de conservação ao fracasso.

A teoria do nicho é uma das propostas da literatura que podem explicar a riqueza de espécies e deve ser observada quando se pensa em conservação ambiental. Ao longo dessa tese, vimos que a riqueza de espécies de grilos respondeu ao aumento do tempo de regeneração florestal, a cobertura de dossel e a profundidade da serrapilheira. Como previsto na literatura, florestas tropicais maduras são densas e com estratos definidos, implicando em diminuição da luminosidade no solo. Menor entrada de luz, aliada a uma mata densa, conserva a umidade alta por mais tempo e a mantêm o microclima estável. Umidade alta e microclima estável são requisitos reprodutivos para muitas espécies de grilos que habitam florestas tropicais. Por isso, nossa medida de cobertura de dossel, pode, na verdade, representar outras dimensões do nicho, como umidade e microclima estável, o que possibilita a sobrevivência e reprodução de espécies com requisitos mais específicos.

O aumento da riqueza de espécies de grilos a profundidade de serrapilheira pode ser devido, especialmente, a dois fatores: alimento e abrigo. Como grilos são

onívoros, uma serrapilheira maior pode conter diferentes fontes alimentares para esses organismos. Maior profundidade de serrapilheira pode, também, representar maiores possibilidades de abrigo contra predadores. Ou seja, uma serrapilheira profunda pode possibilitar a fuga vertical, além da horizontal.

A disponibilidade de recursos é uma dimensão de nicho muito importante para grilos de serrapilheira florestal. Mostramos no segundo capítulo da tese que os grilos estão limitados pela disponibilidade de recursos no ambiente florestal. Isso é particularmente instigante, pois o nosso experimento foi feito em uma floresta intacta, nativa, portanto, essa deve oferecer todos os recursos que lhe são possíveis. Como grilos são onívoros, seria de se esperar que não fossem limitados por recursos, uma vez que podem se alimentar de ampla gama de alimentos. Entretanto, necessidades específicas, como açucares, pode ser o responsável pelo padrão encontrado, especialmente porque usamos caldo de cana em nosso experimento. Quando os grilos identificam uma fonte de açúcar no ambiente, se deslocam até a fonte e consomem o recurso. Esse deslocamento pode ser o responsável pelo aumento da riqueza, pois a agregação aumenta a chance de detectar espécies raras, que são mantidas no ambiente em baixas densidades. Portanto, a disponibilidade de recursos alimentares é um importante mecanismos que pode levar ao sucesso ou insucesso na tentativa de conservação ambiental. Recentes trabalhos, especialmente os publicados por José Alexandre Felizola Diniz-Filho (Universidade Federal de Goiás) e Jean Paul Metzger (Universidade de São Paulo), tem corroborado nossos estudos, especialmente porque enfocam a necessidade do conhecimento de várias dimensões do nicho das espécies, especialmente recursos, condições e área geográfica.

A composição de espécies é um fator que merece ser levado em conta quando se avalia a riqueza de espécies em função de variáveis ambientais. Isso porque, no primeiro capítulo da tese, ficou claro que mesmo após a estabilização no aumento

da riqueza da espécies (atingiu pool regional) a composição de espécies continuou mudando. Isso pode refletir a extrema necessidade de algumas dimensões de nicho para algumas espécies, ou seja, algumas espécies só vão ocupar um determinado local quanto todos os seus requisitos e necessidades forem atendidos. É só nesse estágio que esta poderá competir com as espécies já existentes e permanecer no ambiente.

Mecanismos metodológicos também pode ser responsáveis pela não detecção de algum padrão ambiental, e portanto pela aceitação da hipótese nula quando ela é falsa. Nossos estudos ajudaram a propor uma metodologia mais eficaz para a coleta de grilos de serrapilheira, além de ser apropriada para a conservação de espécimes para análises moleculares. Com a utilização do álcool combustível, espécies que raramente eram amostradas (*singletons*) passaram a ser amostradas efetivamente, possibilitando o melhor conhecimento do pool espécies do local estudado. Além disso, a conservação eficaz do espécimes para análises moleculares e demais estudos, é um quesito ético importante, especialmente em tempos de grande extinção de espécies, especialmente pelas mudanças provocadas pelo homem.

Portanto, existem vários mecanismos que podem afetar a diversidade de espécies. Além dos mecanismos inerentes as próprias espécies, como recursos alimentares, abrigo, microclima, umidade, etc., a correta coleta de dados para a tomada de determinadas decisões também é de suma importância. Dessa forma, essa tese contribui com a comunidade científica por apresentar novos aspectos e/ou dimensões de nicho das espécies que podem ser fundamentais para a conservação ambiental. Além disso, propõe uma metodologia eficaz para a captura de grilos, mais barata e ética, permitindo a utilização dos espécimes coletados para estudos posteriores, especialmente no ramo da biologia molecular.